ASCOSPORE FORMATION IN SACCHAROMYCES

THE EFFECT OF CERTAIN ENVIRONMENTAL FACTORS ON ASCOSPORE FORMATION IN *SACCHAROMYCES CEREVISIAE* (MEYEN) REESS

By

James Calvin, B.A.

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AUTHOR: James Calvin, B.A. (McMaster University)

SUPERVISOR: Dr. J.J. Miller, Associate Professor of Botany

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SCOPE AND CONTENTS: Certain problems relating to the formation of ascospores by baker's yeast were investigated. The effect of acetate concentration and cell population density on ascospore formation and optima were determined. An effort was made to determine factors responsible for some unpredictable variation in ascospore abundance. Certain chemicals were evaluated as sporulation stimulants with results that may be of value in yeast taxonomy. The effect of adding sporulation stimulants to both presporulation and sporulation media was also studied.

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INTRODUCTION

Yeasts have become an increasingly important subject of genetic investigations. Ease of culture, speed of growth and industrial significance are factors contributing to the developing interest in yeast genetics.

Prior to the observations of Schwann in 1839 (24) confusion reigned as to whether the yeasts were fungi or algae. He observed that yeast cells would form new interior cells and that these were liberated by the bursting of the parent cell. Later de Seynes (5) and Reess (18, 19) defined the morphology of these interior cells more clearly. It was Reess who showed that their development resembled the development of the endospores of the lower Ascomycetes. Therefore he termed these cells ascospores and the mother cells asci. He also was the first to observe that yeasts sporulate well on boiled carrots and on slices of other vegetables. Reess however, did not realize that these cells were formed sexually. De Bary (4) postulated that the ascospores were sexual in nature. Finally Satava (22, 23) and Winge (23) described the process of diploidization in *Saccharomyces*, observing that cell fusion occurs immediately after germination of the ascospores. Later Winge (29) proved the existence of haplophase and diplophase generations in *Saccharomyces*.

De Seynes, Reess and Hansen developed various methods for inducing yeast cells to sporulate. Hansen (11) transferred vegetative cells grown in nutrient broth to blocks of gypsum or plaster of paris, a method originally devised by Engel (9). This method is still widely employed. More recently Mrak, Phaff and Douglas (14) induced sporulation on a medium containing the juices of cucumber, carrot, potato and beet mixtures, an improvement of Reess's vegetable wedges. indegren and Lindegren (13) developed a medium consisting of glycerine, calcium carbonate, grape juice and the extracts from beet leaves and roots and from dried yeast. Ascospores were found to develop in a few weeks on this medium, but if spores are desired sooner, plaster of paris blocks are used as a supplement by many workers.

Stantial (25) was able to induce sporulation by adding orange, lemon, grapefruit, lettuce or tomato juices to washed yeast cells in a water suspension. Filtered grapefruit juice gave the most satisfactory yields of asci, up to 90-95% sporulation, most cells having sporulated by the end of 6 days incubation at temperatures between 17-30° C. Stantial (26) determined the active ingredient of the juices to be a sugar. She then used various sugar solutions at varying concentrations and compared their effects on sporulation. Mannose and maltose gave the highest yields, 70-80% sporulation. At the same time she employed a number of chemicals including potassium and sodium chloride, sodium propionate and acetic acid. Later she found that certain salts of acetic acid were highly stimulating. Stantial (25, 26) and Elder (7, 8, 9) who continued her work chose a sporulation medium containing 2 mg. of dextrose and 15-20 mg. of acetate per 20 million cells.

Elder suggested the following detailed sporulation technique: The cells are cultured in flasks containing a filtered heat sterilized tomato juice as a presporulation medium. The cells are freed from the medium by filtering and washing or by centrifuging and washing. Then the cells are suspended in sterilized distilled water, the suspension being adjusted to a concentration of 4 million cells per ml. and diluted 1:1 with the acetate-dextrose sporulation medium. This technique has a great advantage over procedures involving complex natural sporulation media since the nutritional environment can be accurately controlled. Adams (2) modified the Stantial-Elder technique by using solid media and it is his method which is the basis of the present work.

One purpose of this investigation was to determine the effect on ascospore formation of varying cell population densities in sodium acetate solutions. Elder (9) and Adams (2) have reported that sporulation varies greatly with the number of cells per ml. of a given sporulation medium, indicating suitable densities of cell population for obtaining high yields of asci. Adams (2) also found that the optimum number of cells per ml. of sporulation medium varied with different strains.

Stantial (25, 26) and Elder (7, 8, 9) concluded that a relationship seemed to exist between the number of cells and the amount of acetate and dextrose in a liquid culture. In their view the actual volume of liquid is not so important as the ratio of the concentration of these chemicals to the number of cells. Miller and Hellman (15) utilizing agar slants containing varying concentrations of acetate failed to obtain evidence of such a relationship with 2 yeast strains studied. They were able to ascertain that ascospore formation was more frequent at the lower cell

population densities in the presence of 0, 0.01 and 0.1% sodium acetate. Stantial employed liquid sporulation media and Miller and Hellman's results with solid media made it advisable to repeat some of her work employing acetate solutions.

Another object of the present undertaking was to study ascospore formation with respect to its constancy. Two motives were involved: (1) to find strains with high and low sporulation tendencies to be used in genetic crosses (2) to determine the amount of variation under apparently uniform conditions of a given strain. The results of genetic crosses could only be valid if strains did not show too much variability with respect to their sporulation tendencies.

Elder (9) found variability to be a problem in her work and performed experiments to determine whether certain phases of her techniques were responsible, but without success. The writer also has noted unexpected variations in sporulation. In an attempt to determine whether the cells were sensitive to some factor in the environment or to some step in the technique certain experiments were carried out. The studies of previous workers indicate that temperature, p.H. and moisture over a wide range are not critical. Therefore these were not studied further, but instead a number of other factors were investigated.

The final purpose of the present research was to study the effect of various chemicals on the sporulation of the F.J. strain, and incidentally to develop new sporulation media. Such a task is worthwhile in that no universal sporulation medium has been developed for yeast. A medium which stimulates sporulation with one species or genus may be unsuitable for another. Thus the development of new sporulation media would aid research

in yeast taxonomy and genetics. Also in the future, such work as this may contribute to an understanding of the sporulation process.

METHODS AND MATERIALS

The yeast strain employed for the most part in these studies has been designated as strain F.J. by previous workers in the Department of Biology, McMaster University. It was isolated from packaged Fleischmann's yeast. Other cultures used during the course of experimentation were isolated by the dilution plate method. Pour plates were made using high dilutions of Fleischmann's dried yeast pellets. Commercial yeast normally contains bacteria and to prevent their growth streptomycin was added to the plating medium.

The procedure followed for obtaining ascospores was based on Adams' (2) modification of the Stantial-Elder method. Cells were allowed to multiply for 48 hours at 27° C. on a solid presporulation medium. The cells were then removed from the presporulation medium by an inoculating needle and suspended in sterilized distilled water. The suspension was then adjusted to the appropriate cell population density with the aid of a haemocytometer. Five ml. of the cell suspension were then pippetted into 5 ml. of a sporulation medium of twice the desired concentration. After mixing, 0.8 ml. of the sporulation culture was pippetted into sterilized vials, 15 mm. in diameter and 45 mm. in length. The vials were then incubated at 27° C. for 7 days, at which time sporulation percentages were determined.

The above technique of course involved a liquid sporulation medium. A much simpler procedure also employed involved the use of a solid sporulation medium. Here the cells were simply transferred by an inoculating

needle,) from presporulation slants to sporulation slants which were then incubated for 7 days.

The percentage of cells that had formed asci was estimated by examining 200 cells from each vial or slant. All asci included in the results are listed as being 2- or 4- spored, examples of both types are given in Figs. 1 and 2. One- and 3-spored asci were not counted since 1-spored asci might be mistaken for a vegetative cell while a 3-spored asci might easily contain a fourth spore hidden beneath; hence the latter were counted as 4-spored asci.

In the experiments to follow, 5 replicates were always employed unless otherwise stated. Also, unless otherwise stated, a solid presporulation medium was used followed by a liquid sporulation medium.

The terms presporulation medium and sporulation medium will be used frequently in the work to follow. These may be defined as follows: (1) Presporulation medium--any medium on which cells are propogated prior to transferring to a sporulation medium, (2) Sporulation medium--any medium to which cells are transferred in order to cause ascospore formation.

The presporulation media employed were:

(1) A solid medium in which 5% dextrose and 1% yeast extract(Difco) were added to nutrient agar (Difco).

(2) Yeast Nitrogen base agars--prepared by adding 2.5% bacto agar to the yeast nitrogen base liquid medium developed by Wickerham (28) and sold commercially by the Difco Corporation. This medium which lacks a carbon source was developed for carbon assimilation tests in yeast taxonomy.

All presporulation media were in a solid form. The media were dispensed in 5 ml. quantities in test tubes. The presporulation medium most commonly used was that containing dextrose and yeast extract.

The sporulation media used were:

(1) Sodium acetate solutions--prepared by adding weighed amounts of sodium acetate to an appropriate volume of distilled water. For the most part a 0.1% sodium acetate solution was used.

(2) Acetate agar--2.5% bacto agar was added to a 0.1% sodium acetate solution. In one experiment other salts of acetic acid were studied.

(3) Solutions of fructose, glucose, mannose, glucose 1-phosphate, fructose 1, 6-diphosphate, histamine, glucosamine and potassium dihydrogen phosphate.

(4) The foregoing solutions were prepared as solid media as well by the addition of 2.5% bacto agar.

Control tubes or vials were included in each experiment. If the sporulation medium was solid, 2.5% bacto agar was used as a control. If the sporulation medium was a liquid, distilled water served as the control.

EXPERIMENTAL

(A) EFFECT OF LIGHT ON ASCOSPORE FORMATION

Adams (3) performed a number of experiments comparing sporulation in darkness and diffuse light and found no important difference. Other than this work, the effect of light on sporulation has not been studied at this university.

In this preliminary experiment, sets of vials containing sporulation cultures were placed under varying light conditions (a) continuous darkness (b) continuous light from a 25 watt bulb (c) near a north window where conditions of direct sunlight never prevailed (d) near a south window where the cultures were exposed to direct sunlight in the morning.

Table I shows the sporulation percentages obtained under the various

TABLE I

Ascospore formation under 4 conditions of illumination.

Light Conditions	Per cent yield of Asci
Continuous darkness.	19.2*
Light from a north window. No direct sunlight.	28.8
Continuous light from a 25 watt bulb.	15.4
Light from a south window. Direct sunlight in the morning.	10.2

*Each figure based on an examination of 1000 cells; 200 from each of 5 vials.

L.S.D. = 6.5 for P 0.05.
9.1 for P 0.01.

light conditions.

The results demonstrate that cells exposed to strong direct light are less apt to sporulate than are cells which are never exposed to light during the sporulation period.

(B) THE RELATION OF THE CELL POPULATION DENSITY AND OF THE ACETATE CONCENTRATION TO THE FREQUENCY OF ASCOSPORE FORMATION.

Cells obtained from slants of 5% dextrose nutrient agar containing 1% yeast extract were placed in the following concentrations of sodium acetate: 3.0, 1.0, 0.3, 0.1, 0.03 and 0.01%. With each concentration 9 cell population densities were used: 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 million cells per ml. Since the densities were adjusted with a haemocytometer some innacuracy may be expected.

The results of this experiment are summarized in Fig. 3. The cell population density of 4 million per ml. seemed optimum for ascospore formation regardless of the acetate concentration. Other workers, Tremaine (27) and Adams (2) ascertained an approximate value of 1 million cells per ml. to represent an optimum during the course of their studies. The optimum acetate concentration would appear to be 0.1 or 0.01%. Adams (1) and Elder (7, 8, 9) show a preference for the former figure while Fowell (10) has recently obtained optimum sporulation in his strains with a 0.3% acetate concentration.

Of interest is the frequency of 4-spored asci throughout the experiment as shown in Fig. 4. In general the trends in Figs. 3 and 4 are more or less comparable. Only about half of the sporulating cells formed 4-spored asci. Tremaine (27), has shown that by modifying the presporulation medium the ratio of 4-spored asci/total number of asci, can be varied.

(C) STUDIES OF VARIATIONS IN THE ABUNDANCE OF ASCOSPORES UNDER APPARENTLY UNIFORM CONDITIONS.

During the work inconsistencies in yields were sometimes found from one experiment to another, using cells from the same storage culture and keeping sporulation conditions as uniform as possible. Experiments in this section represent an effort to account for these inconsistencies

(1) Variation in ascospore formation among yeast isolates. Adams (3) has suggested that 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 presumably single cell isolates. An attempt has been made to evaluate this variation with the genetical factor in mind.

Dilutions of yeast cells from a Fleischmann's yeast cake were plated on 5% dextrose nutrient agar containing streptomycin and 1% yeast extract. Thirty-seven isolations were made to slants of 5% dextrose nutrient agar 1% yeast extract and subcultured once. Sporulation cultures were then prepared. Four experiments were performed, each with 7 to 10 isolates.

Table II indicates that the ability to form ascospores varied a great deal among the isolates. Several isolates yielded relatively few asci while one isolate yielded 50% asci. Of the 37 isolates, 7 yielded 30-35% asci. However, these values were obtained totally from the last two experiments where yields were notably higher.

Representative cultures from the foregoing isolates were checked for their sporulation constancy one month later. Table II summarizes the results. Obviously the high and low sporulating isolates tended to give ascospore yields that showed less diversity than in the previous experiment.

A further experiment in the study of variability involved a comparison

TABLE II

Variation in asci yields of 37 isolates from a yeast cake.

Isolations from Plating #1			Isolations from Plating #2		
Isolate No.	Initial per cent yield of asci.	Second per cent yield of asci.	Isolate No.	Initial per cent yield of asci.	Second per cent yield of asci.
1	18.5*		11	1.3**	
2	0.6		12	16.5	
3	2.7		13	8.3	
4	16.8		14	0	
5	13.8		15	8.1	
6	14.4		16	0.4	
7	8.2	23.8*****	17	4.4	
8	Contamination		18	4.9	
9	5.1		19	14.0	
10	7.6		20	8.2	

Isolations from Plating #3			Isolations from Plating #4		
Isolate No.	Initial per cent yield of asci.	Second per cent yield of asci.	Isolate No.	Initial per cent yield as asci.	Second per cent yield of asci.
21	32.2***		28	22.6****	
22	30.1		29	19.5	
23	15.4		30	0.1	37.5
24	50.3	42.5	31	0.1	
25	30.2	31.4	32	34.8	
26	21.0		33	26.1	
27	33.9	30.0	34	31.7	47.0
			35	30.2	38.2
			36	7.1	
			37	17.7	37.0

Each figure based on an examination of 1000 cells; 200 from each of 5 vials.

* L.S.D. = 6.7 for P 0.05
9.1 for P 0.01

** L.S.D. = 7.3 for P 0.05
9.9 for P 0.01

*** L.S.D. = 4.3 for P 0.05
5.8 for P 0.01

**** L.S.D. = 2.7 for P 0.05
5.0 for P 0.01

***** L.S.D. = 5.5 for P 0.05
7.4 for P 0.01

of the variability shown by 10 isolates, presumably arising from single cells, from a single Fleischmann's yeast package and that shown by subcultures from one of these single cell isolates. Ten isolations were made from dilution plates containing 5% dextrose nutrient agar plus yeast extract and streptomycin to presporulation slants numbered 1 to 10. Six hours after inoculating slant number 1 transfers were made from it to 10 other presporulation tubes, these sub-isolates being named 1(a), 1(b), etc.

Table III summarizes the results, which show indications in both cases of variation. However, variation was not nearly as pronounced as in the previous experiments.

(2) Effect of time of incubation on ascospore formation.

The problem investigated here was to relate the percent sporulation to the time of incubation of sporulation cultures with a view to determining whether it is a factor in causing varying yields.

In this experiment 20 ml. quantities of sporulation culture suspension were placed in 250 ml. Erlenmeyer flasks. Fig. 5 indicates that maximum sporulation was reached in 4 days. The results show that the 7-day incubation time used in these studies, while longer than necessary, should not be a factor in the variation in ascospore yields since the curve has flattened out long before this point is reached.

Adams (2) noted no differences in sporulation percentages for a single sporulation culture after periods of 7 and 14 days, which is in agreement with the foregoing.

(3) The effect on ascospore formation of washing cells.

During the course of experimentation Ascospores were occasionally found in the distilled water controls. It was thought that perhaps some dextrose carried over from the presporulation medium with the inoculum

TABLE III

A comparison of the variation shown by 10 distinct isolates and that shown by 10 subcultures from a single isolate.

Isolate No.	Per cent yield of asci	Isolate No.	Per cent yield of asci
1	33.4*	1(a)	33.1*
2	31.1	1(b)	33.2
3	20.2	1(c)	29.0
4	29.5	1(d)	29.4
5	19.0	1(e)	27.1
6	30.9	1(f)	29.0
7	30.7	1(g)	25.9
8	Contamination	1(h)	39.6
9	33.4	1(i)	25.8
10	29.9	1(j)	41.0

*Each figure based on an examination of 1000 cells; 200 from each of 5 vials.

L.S.D. = 7.1 for P 0.05
9.6 for P 0.01.

might be the cause of this sporulation. Stantial (25, 26) and Adams (1, 2, 3) have shown that dextrose in the appropriate concentration does stimulate ascospore formation. Now, if the cells are washed presumably all dextrose would be removed from their surfaces.

A cell suspension was divided into two parts, one part of the suspension being washed, the other not. The washing of cells involved centrifuging the cells for 5 minutes, the supernatant being poured off. Sterile water was then added and a new suspension formed. After a second centrifuging the supernatant was again poured off and the cells were resuspended.

The results as presented in Table IV show that the washing of cells did not affect sporulation either in distilled water or in acetate solution.

(4) The effect of impurities in water on ascospore formation.

This experiment was performed to determine whether impurities in the distilled water might affect sporulation significantly.

Sporulation cultures with and without sodium acetate were prepared using 4 sources of water (1) distilled water from a tin lined Barnstead still; (2) water from this still passed through an ion absorption tube; (3) water from this still distilled twice through a glass still; (4) tap water. Cells to be placed in each of these 4 types of water were first washed in it.

The results of the experiments are shown in Table V. It seems evident that variation in water source had no effect on sporulation. Thus it is unlikely that impurities in the distilled water used throughout the experiments are a cause of variability. No appreciable sporulation was noted in the absence of acetate in this experiment.

TABLE IV

Ascospore formation by washed and unwashed cells in distilled water and 0.1% acetate solution.

Treatment of Cells	Per cent yield of asci	
	0.1% Na. acetate	Distilled water
Washed	27.6*	14.5
Unwashed	27.1	16.9

*Each figure based on an examination of 1000 cells; 200 from each of 5 vials.

L.S.D. = 6.0 for P 0.05
7.7 for P 0.01

TABLE V

Sporulation in 0.1% acetate solution with water variously treated.

Water Source	Per cent yield of asci
Distilled water from Barnstead still	11.9*
Distilled water from Barnstead still passed through an ion absorption tube	17.3
Distilled water from Barnstead still twice distilled through a glass still	17.4
Tap water	12.7

*Each figure based on an examination of 1000 cells; 200 from each of 5 vials.

L.S.D. = 3.7 for P 0.05
5.0 for P 0.01

(D) THE EFFECT OF VARIOUS CHEMICALS ON ASCOSPORE FORMATION.

(1) The evaluation of certain chemicals as sporulation stimulants.

Eleven chemicals were used in the first experiment. Some have been used as sporulation stimulants by other workers. Stantial (25, 26) and Adams (1, 2, 3) studied glucose, mannose, fructose and acetate while Ronsdorf (20) showed that histamine induced the production of zygospores in Phycomyces blakesleanus. Stantial (25, 26) also used dihydrogen potassium phosphate. Other chemicals such as glucosamine, glutaric acid and phosphorylated sugars are being utilized for the first time in the study of sporulation in Saccharomyces to the writer's knowledge.

Ten chemicals were added to distilled water in the concentrations shown in Table VI. Sporulation cultures were made with each of the 10 media, the results being tabulated in Table VI.

Glutaric acid, glucosamine and glucose promoted little if any sporulation. Also there is slight evidence of stimulation of ascospore production by histamine and fructose as compared with the distilled water control.

With respect to the sugars used, mannose gave most promise as a sporulating agent, exceeding the effect of glucose and fructose. Stantial (26) suggested mannose as being the best sporulating medium among the sugars used by her. The effects of glucose and fructose, however, are contrary to their effects as noted by Stantial (26) and Adams (1).

The phosphorylated sugars gave the highest yields. Since in respiration sugars must first be phosphorylated such results might be expected. Stantial (25, 26) found that potassium dihydrogen phosphate did not enhance ascospore formation. The results here support this since the combination of acetate and dihydrogen potassium phosphate caused no increase in yield over the acetate medium alone.

TABLE VI

The effect of various liquid sporulation media on the per cent yield as asci.

Sporulation medium	Presporulation medium	
	5% Dextrose nutrient agar	5% Dextrose nutrient + 1% yeast extract
0.01% Fructose	1.9*	6.6**
0.05% Fructose	0	0
0.01% Glucose	0	0
0.05% Glucose	0	0
0.01% Mannose	2.4	11.5
0.05% Mannose	0.3	0.3
0.01% Histamine	0	3.5
0.05% Histamine	0	0.4
0.01% Glutaric acid	0	0
0.05% Glutaric acid	0	0
0.01% Glucosamine	0	0
0.05% Glucosamine	0.2	0.1
0.01% Glucose-1-phosphate	10.6	22.8
0.05% Glucose-1-phosphate	7.8	27.8
0.01% Fructose-1, 6-diphosphate	3.9	11.4
0.05% Fructose-1, 6-diphosphate	5.9	21.8
0.1% Na. acetate + KH_2PO_4	4.2	13.6
0.1% Na. acetate	1.5	14.1
Distilled water	1.0	7.6

Each figure based on an examination of 1000 cells; 200 from each of 5 vials.

* L.S.D. = 2.0 for P 0.05
2.7 for P 0.01

** L.S.D. = 5.3 for P 0.05
7.2 for P 0.01

Table VI also shows the effect of adding yeast extract to the presporulation medium. It greatly increased sporulation when present, as already noted by Tremaine (27).

The effect of some of the chemicals was investigated further in another experiment in which glucose, fructose, mannose, the 2 phosphorylated sugars, sodium acetate, dihydrogen potassium phosphate were used in solid sporulation media. In this experiment the range of concentrations was expanded and only 3 replicates were used.

The results are shown in Fig. 6. It can be seen that glucose, mannose and fructose behave similarly regarding their effect on ascospore formation. The optimum for all 3 was a 0.0028 molar (0.05%) concentration in water agar which corresponds to the figures mentioned by Elder (7, 8, 9) and Adams (2). Little sporulation occurred above 0.028 molarity (0.5%). It is interesting to note that mannose gave the highest sporulation of the 3 sugars. Fructose also yielded high values while glucose was the least stimulating. It is not clear why fructose and glucose showed less satisfactory results as solutions in the preceding experiment.

The phosphorylated hexose sugars yielded different results than did the hexoses. All concentrations for the hexoses and their phosphorylated derivatives are expressed in terms of molarity and thus the effect of equal numbers of molecules can be compared. Although the phosphorylated sugars did not give as high yields as mannose, glucose and fructose, highest sporulation occurred at the higher concentrations of the former where the yield from the hexoses was low.

In Table VII the effect of KH_2PO_4 in solid agar is studied. Obviously the claim by Stantial (25, 26) that dihydrogen potassium phosphate does not

TABLE VII

The effect of acetate and KH_2PO_4, in a solid medium, on ascospore formation.

Sporulation medium	Per cent yield of asci.*
Water agar + 0.1% KH_2PO_4	3.5
Water agar + 0.1% glucose + 0.1% KH_2PO_4	4.1
Water agar + 0.1% glucose	2.33
Water agar + 0.1% Na. acetate	26.8
Water agar	2.2

*Each figure based on an examination of 600 cells; 200 from each of 3 slants.

stimulate sporulation is substantiated.

The effect of glucose-1-phosphate was studied further in an effort to determine the concentration which gives maximum sporulation. Table VIII indicates that the range of concentrations from 0.1 to 1.6% glucose-1-phosphate induced about 10% sporulation with no clear optimum. The per cent yield of asci in this experiment was not as high as in the preceding, but it is noteworthy that ascospore formation occurred at the high concentrations. Presumably a concentration of 0.72% glucose-1-phosphate in water agar would yield satisfactory results as a sporulation medium since this concentration gave the highest yields in the previous experiment and was one of the 2 concentrations to give the maximum asci yield in this experiment.

(2) The effect of adding certain chemicals to the presporulation and sporulation media.

This experiment was designed to determine whether contact with a chemical prior to being exposed to it in the sporulation medium is stimulating to sporulation. The chemicals studied were dextrose and the sodium, ammonium and magnesium salts of acetic acid.

The presporulation medium consisted of yeast nitrogen base agar to which the chemical being studied was added in a 1% concentration. In each case cultures were transferred twice on this medium, after 48 hrs. incubation and then placed on water agar containing the same chemical. As a control 5% dextrose nutrient agar containing 1% yeast extract was also used as a presporulation medium.

The results are presented in Table IX. Prominent growth on the yeast nitrogen base presporulation medium occurred only with dextrose and growth was relatively slight with the other carbon sources.

TABLE VIII

The effect of various concentrations of glucose-1-phosphate, in water agar, on sporulation.

Per cent concentration of glucose-1-phosphate in water agar	Per cent yield of asci.*
0.0720	5.5
0.14	11
0.72	11
1	8.2
1.1	7.3
1.3	7.7
1.6	10.5

* Each figure based on an examination of 600 cells; 200 from each of 3 slants.

TABLE IX

Yields of ascospores on 4 sporulation media produced by cells from various presporulation media.

Sporulation medium	Presporulation medium	Growth	Per cent yield of asci.*
Water agar + 0.05% dextrose	**Yeast nitrogen base agar + 1% dextrose.	+ +	1.2
	5% Dextrose nutrient agar + 1% Yeast extract.	+ + + + +	5.0
Water agar + 0.1% Na. acetate	Yeast nitrogen base agar + 1% Na. acetate.	+	11.5
	5% Dextrose nutrient agar + 1% Yeast extract.	+ + + + +	26.0
Water agar + 0.1% Mg. acetate	Yeast nitrogen base agar + 1% Mg. acetate	0	10.3
	5% Dextrose nutrient agar + 1% Yeast extract	+ + + + +	35.8
Water agar + 0.1% NH_4 acetate	Yeast nitrogen base agar + 1% NH_4 acetate	0	0
	5% Dextrose nutrient agar + 1% Yeast extract	+ + + + +	0.17

* Each figure based on an examination of 600 cells; 200 from each of 3 vials.

** Growth on Yeast nitrogen base = 0.

Yields of asci were low with dextrose and ammonium acetate in the sporulation medium. The fact that ammonium acetate did not stimulate sporulation with either of the presporulation media is a point of interest. Saito (21) has shown that ammonia or compounds yielding ammonia are detrimental to sporulation in Saccharomyces under certain conditions.

Yields were greater when magnesium or sodium acetate was present in the sporulation medium. But when either acetate compound served as the carbon source in the presporulation medium the sporulation was greatly diminished. Evidently growth, with the acetate compound in the presporulation medium, does not produce cells which will give as high sporulation yields as with the usual presporulation medium. This is probably due to the poor growth on the yeast nitrogen base presporulation medium with acetate as the carbon source.

About one month after the completion of the experiment minute raised areas of yeast cells appeared on the surface of the yeast nitrogen base agar slants with acetate as the carbon source. These areas resembled the daughter colonies sometimes noted in bacterial cultures and which are ascribed to mutations. Possibly mutations here have given rise to cells able to use acetate more readily as a carbon source. It would be interesting to study the sporulation of cells from these areas on an acetate sporulation medium.

DISCUSSION

It is evident from the preceding work that ascospore formation in acetate solution is conditioned greatly by environmental factors.

In a preliminary experiment light conditions were shown to affect the percentage of asci produced. The results of this experiment are not

conclusive in themselves but suggest that further studies of light as a factor in ascospore formation may prove worthwhile.

The studies relating to cell population density indicate that a density of 4 million cells per ml. approximates the optimum for the F.J. strain regardless of the acetate concentration. The optimum acetate concentration ranged from 0.01 to 0.1%.

Stantial (25, 26) and Elder (7, 8, 9) concluded that the concentration of the acetate was not important regarding sporulation but rather that the ratio between the amount of acetate and the population density determined the extent of sporulation. That is, the cell population density for maximum sporulation would vary with the acetate concentration. The writer's findings are at variance with this view.

Variability of ascospore formation was studied by a series of experiments. Since strong diffuse light was shown to adversely affect sporulation, during the course of experimentation cells were never exposed to such an environment. The period of incubation in a liquid medium used throughout the experiments was studied and the results revealed that this was not a factor which would cause any great variability in sporulation. Likewise impurities in the water source used during experiments were found to cause no important variation in ascospore formation. The fact that cells were not washed during the course of experimentation proved to be unimportant as a factor in causing a strain to vary its sporulation potential.

That variability occurs in the ascospore yields from an isolate under apparently uniform conditions seems evident from the experiments, and before dependable genetical studies can be made on sporulation, such variation must be controlled. The cause of such variation has not been

determined as yet nor has the reason for the sporadic occurence of sporulation in distilled water from time to time been found.

It is the opinion of the author that changes in cell population density were a factor in variability, but certainly other factors, as yet undetermined, were involved. In the future a cell population density (in acetate liquid medium) of 4 million per ml. is suggested as a method of minimizing variability.

In the studies on chemical stimulants to sporulation particular attention was paid to the sporulation medium. Some of the sporulation media studied were not new, having been studied by Stantial (25, 26) and Adams (1, 2, 3). However such studies were useful in that they confirmed for the most part the results of these workers.

To the writer's knowledge the effect on yeast sporulation of phosphorylated hexoses had not been studied previously. Hawker (12) however, has stimulated perithecial production in Melanspora destruens with such sugars. Glucose-1-phosphate and fructose-1, 6-diphosphate were less effective at low concentrations and more effective at higher concentrations in a solid medium than the corresponding hexoses in stimulating ascospore production. The following were found to have little effect in stimulating sporulation at the concentrations used in the experiments: histamine, glutaric acid, glucosamine, and dihydrogen potassium phosphate.

Certain general findings were noted during the experiments and are worthy of discussion. The first has to do with the frequency of formation of 4-spored asci. None of the sporulation media, even the phosphorylated sugars, produced as many 4-spored asci as did the 0.1% sodium acetate solution or agar, nor were they as consistent in ascospore production.

Secondly, sporulation in distilled water is not additive. That is,

sporulation in distilled water when it occurs is not correlated with high sporulation in acetate solution. Also sporulation never seemed to occur to any degree on water agar. Probably the most important point regarding sporulation in distilled water is that it did not occur at very high cell population densities nor at extremely low cell population densities but generally in the range 4 to 0.5 million cells per ml.

A third point of interest is the fact that from time to time 4-spored asci seemed characterized by the fact that the mother cell had started to bud. In some experiments almost every 4-spored ascus showed this feature while in others this phenomena was absent.

Finally throughout the course of the work the writer has noted that yields of ascospores are generally higher in a solid sporulation medium than in a liquid medium. This point is discussed at length by Adams (1).

Both Tremaine (27) and Adams (3) have discussed the practical aspects of the technique used to obtain ascospores. Both emphasize the suggestion of Pomper (17), that the method would be more advantageous than methods involving complex natural media, since the nutritional environment can be exactly controlled, which is of great importance in the study of the inheritance of adaptive enzymes in yeasts. The writer found the main asset of the method to be its simplicity, a feature which Phaff and Mrak (16) found lacking in the Stantial-Elder technique. This simplicity in technique was of value in studying sporulation stimulants.

For high ascospore yields with a liquid sporulation medium the writer suggests a change in technique, substituting a cell population density of 4 million per ml. in place of 1 million per ml. as used by Adams, (1,2,3) and Tremaine (27).

SUMMARY

1. Strong direct light apparently tended to inhibit sporulation.

2. The optimum cell population density for sporulation in acetate solution was of the order of 4 million per ml. This was not affected by variations in the acetate concentration.

3. The optimum sodium acetate concentration of the liquid sporulation medium was found to be 0.1%. This was not affected by variations in the cell population density.

4. Variability in sporulation occurred not only among isolates from a single yeast pakcage but also among sub-cultures of a single isolate. The factors behind this variability were not found.

5. Fructose-1, 6-diphosphate and glucose-1-phosphate were found to yield asci at high concentrations, being less effective at low concentrations and more effective at higher concentrations than the corresponding hexoses.

6. Exposure of cells to acetate in the presporulation medium prior to their contact with it in the sporulation medium did not increase sporulation.

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PLATE I

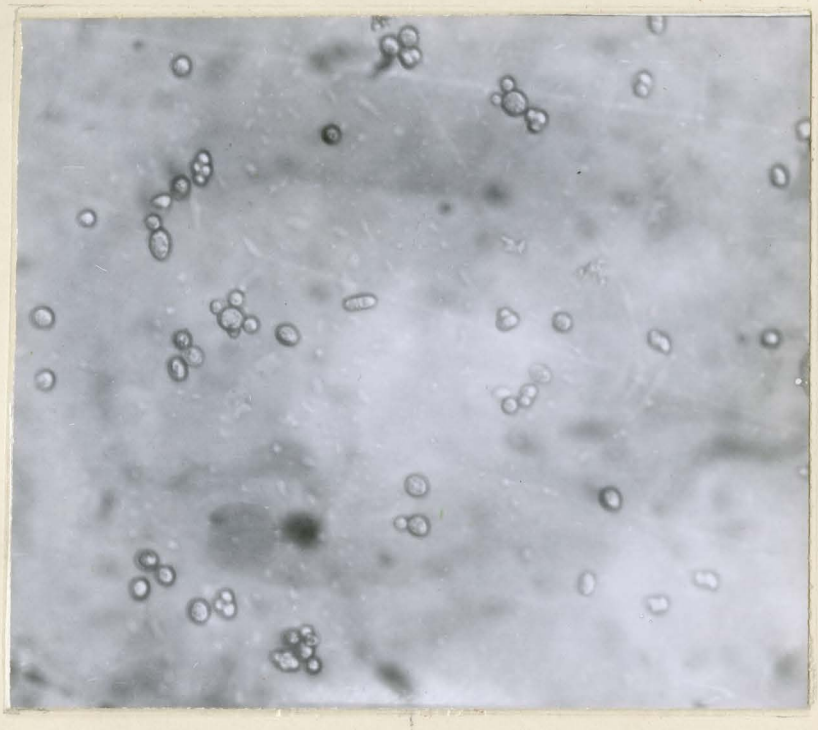

Fig. 1. Cells from a 7 day culture on water agar + 0.1% sodium acetate showing 2-spored and 4-spored asci. x 1500.

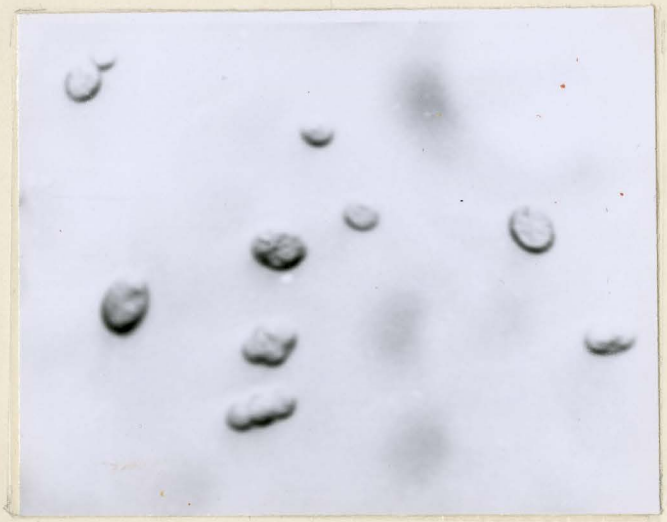

Fig. 2. Cells from a 7 day culture on water agar + 0.1% sodium acetate showing a 3-spored and a 4-spored ascus. x 3000.

PLATE II

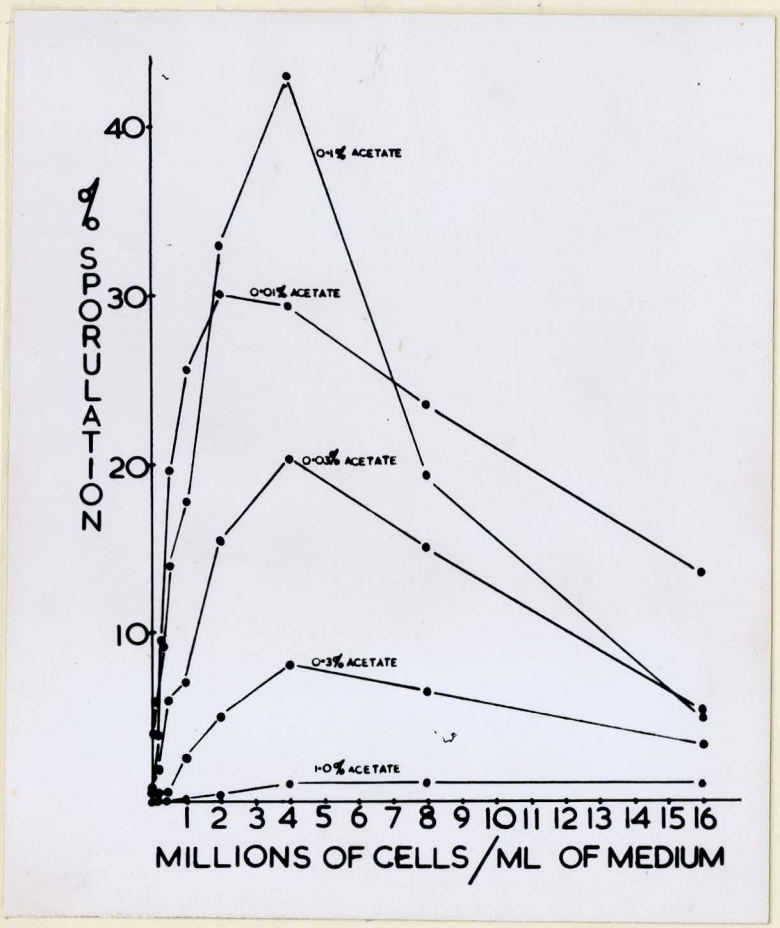

Fig. 3. The effect of cell population density and sodium acetate concentration on sporulation.

PLATE III

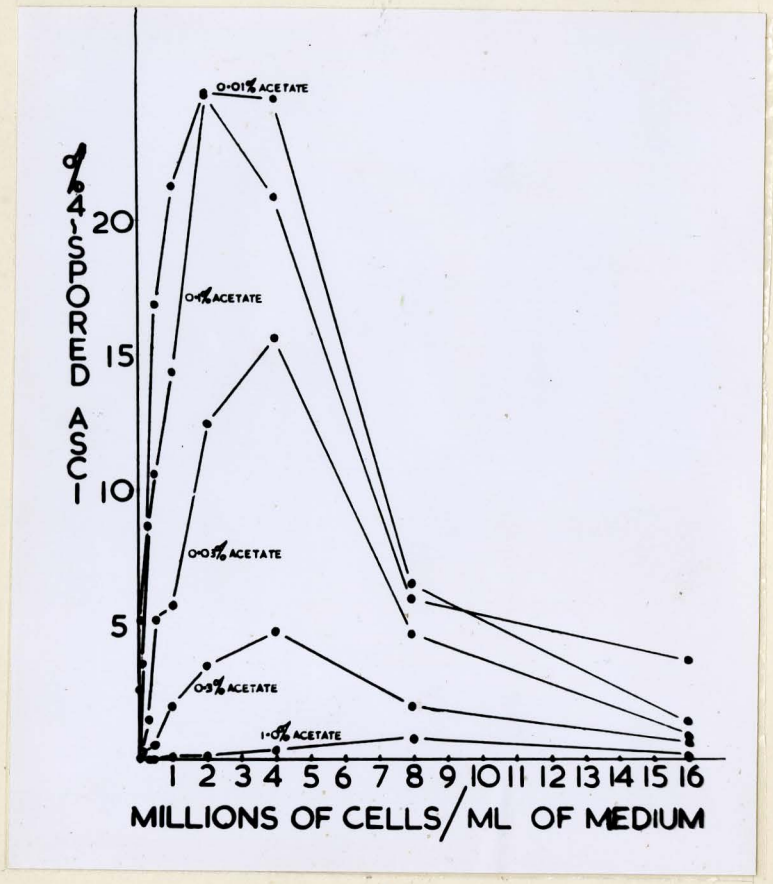

Fig. 4. The effect of cell population density and sodium acetate concentration on the production of 4-spored asci.

PLATE IV

Fig. 5. Abundance of asci present in 0.1% sodium acetate solution during a period of 21 days after inoculation.

PLATE V

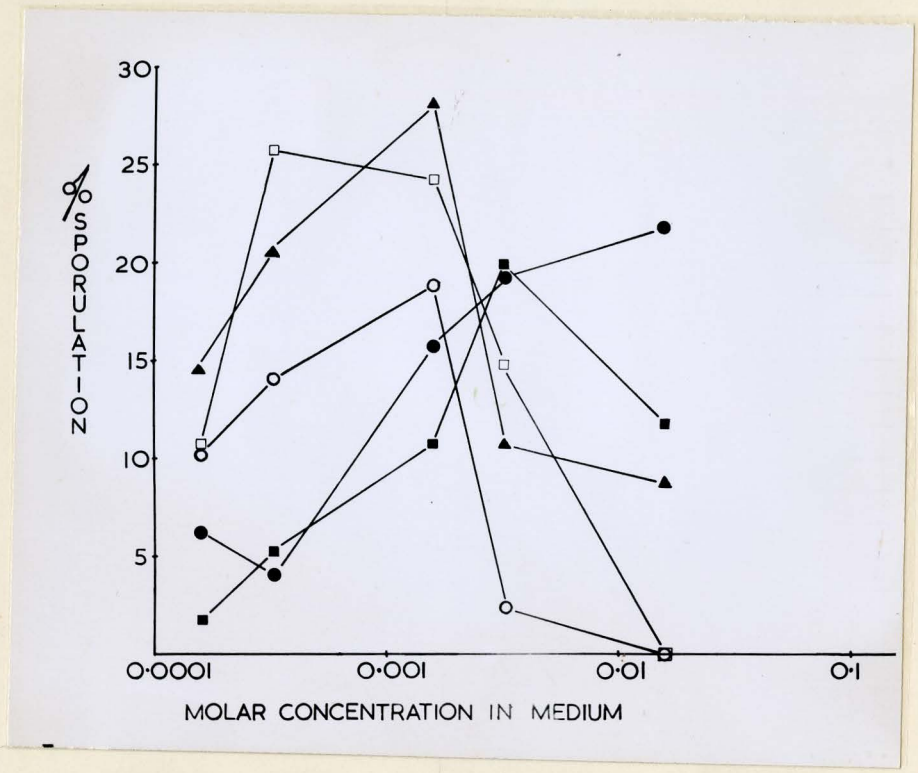

Fig. 6. Abundance of asci after 7 days on concentrations of hexoses and hexose phosphates. ○ glucose, ● glucose-1-phosphate, □ fructose, ■ fructose-1, 6-diphosphate, ▲ mannose.