THE EFFECT OF CERTAIN NUTRIENTS ON ASCOSPORE FORMATION IN *SACCHAROMYCES CEREVISIAE* MEYEN

by

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TABLE OF CONTENTS

The Effect of Certain Nutrients on Ascospore Formation in *Saccharomyces cerevisiae* Meyen

INTRODUCTION

Klebs (11), one of the early workers on the physiology of the reproduction of fungi, made four generalizations concerning the reproduction of fungi which were based on his own work and on that of others. (1) Growth and reproduction depend on different conditions in all organisms; the external conditions mainly determine whether growth or reproduction will occur in lower organisms. (2) A fungus will not reproduce if conditions are favourable for growth. The conditions favourable for reproduction are unfavourable for growth. (3) Growth may occur under a wider range of conditions than does reproduction. (4) Growth, or rather the period of assimilation accompanying growth, is a preliminary step for reproduction since it creates a suitable environment for reproduction.

Both physical and nutritional factors affect the reproduction of fungi. Nutritional factors known to influence reproduction in culture are: nitrogen source, carbon source, carbon-nitrogen ratio, trace elements,

concentration of nutrients, specific reproductive factors, and vitamins (14). The critical study of the effect of vitamins on growth and reproduction has become possible only in the last few decades, when the synthesis of vitamins allowed the use of preparations of a given vitamin free from contamination by others.

Although the dependence of a number of filamentous fungi on an exogenous source of certain vitamins for vegetative growth has been well established, little is known concerning their vitamin requirements for sexual reproduction. Hawker (8) found that Melanospora destruens, which required biotin for growth, required thiamin in addition to biotin for perithecial formation. Robbins and Ma (25) interpreted the failure of Ceratostomella pluriannulata to form perithecia in the absence of thiamin, which was required for growth, as being due to a general physiological disturbance causing the mycelium to lack sufficient vigor to support sexual reproduction. Barnett and Lilly (3) and Lilly and Barnett (12) reported that the number of perithecia formed by Sordaria fimicola, which required biotin for growth, was a function of the biotin concentration in the medium when sub-optimal biotin concentrations were used. They later reported (12) that growth, perithecial formation, and maturation of asci and ascospores in S. fimicola required increasing concentrations

of biotin in that order. Lilly and Barnett (4) reported similar results with Ceratostomella fimbriata in regard to thiamin which is required for growth of this fungus. They also found that, when thiamin-starved mycelium was placed in water containing thiamin, perithecia formed without marked mycelial development. In a medium in which the nutrients were diluted and the thiamin concentration was high, they found a quantity of mycelium weighing only 2 mg. to be capable of forming perithecia. Lilly and Barnett concluded from these results that the influence of thiamin upon the formation of perithecia appeared to be direct, rather than acting through a general disturbance of the metabolism of this fungus. However, they admitted that the mode of action of thiamin is still not clear, since little is known about the metabolic processes in fungi. Lilly and Barnett (13) obtained similar results with Chaetomium convolutum, which requires thiamin and biotin for growth.

Raper (22), in a recent review of the chemical regulation of sexual processes in the thallophytes, distinguished between two types of reactions. He termed substances affecting sexual reproduction which originated external to the species "ectohormoids". These substances may be present in the culture medium or supplied by other organisms. Substances affecting sexual reproduction which were produced by the affected plant or by other individuals

of the same species were termed hormones. Raper concluded that the influence of "ectohormoids" was not direct, since the entire sexual process was stimulated. In addition, he stated the opinion that marked sexual stimulation by "ectohormoids" reflected sub-optimal production of required substances by the plant's own metabolic processes.

Yeasts, like filamentous fungi, may require certain vitamins for growth. Burkholder (5) concluded that multiple vitamin deficiencies were more common among the yeasts than among the filamentous fungi. The vitamin requirements of 38 species and strains of yeast were reported by Burkholder (5) and for 110 additional species and varieties by Burkholder et al (6). One hundred and fourteen were deficient for biotin, 48 for thiamin, 44 for pentothenic acid, 19 for inositol, 19 for nicotinic acid and 19 for pyridoxine.

The effect of vitamins on ascospore formation in yeasts has not been studied in detail. Such studies would be difficult using the complex natural media commonly employed for inducing sporulation in these fungi. These are: vegetable wedges (Rees (23)); vegetable infusions (Mrak, Phaff, and Douglas (18)); commercial juice of eight vegetables (Henrici (9), Wickerham et al (31)); fruit and vegetable infusions poured over gypsum blocks (Lindegren and Lindegren (15)); peptone, meat-extract agar

(Gorodokowa (7)).

Obviously, a synthetic sporulation medium of known chemical composition is essential when investigating the vitamin requirements for sporulation of yeast. This has been provided through the studies of Stantial (27, 28), who reported that dilute solutions of sugar and acetate, alone or in combination, induced sporulation in Saccharomyces cerevisiae.

Ascospore formation in such a solution, however, has been found to be conditioned by the composition of the presporulation medium in which the yeast cells are allowed to develop vegetatively before being transferred to the sporulation medium. Stantial (28) found that growth from wort gave yields of approximately 70% asci, whereas cells from a mineral salts-dextrose medium gave yields of only 25% when transferred to an acetate solution. The addition of inositol and Bios II* to the latter presporulation medium increased sporulation when the cells were transferred to the acetate solution. Adams (1) reported high yields of asci when cells multiplied on biotic media were transferred to an acetate-dextrose solid sporulation medium. He also found (2) that asci were rarely produced on this sporulation

* (now known to be biotin and pantothenate)

The term biotic will be used to denote media prepared from plant or animal material.

medium by cells multiplied on a synthetic medium formulated by Lodder (17) of the following composition: 2% dextrose, 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and 2% agar in distilled water.

From the foregoing, it is evident that the composition of the presporulation medium, as well as that of the sporulation medium must be controlled if the vitamin requirements for yeast sporulation are to be determined. The purpose of the present investigation was (1) to find a synthetic presporulation medium on which yeast would multiply and then sporulate when transferred to an acetate solution and (2) to use this medium to study the vitamin requirements for ascospore formation in yeast.

METHODS AND MATERIALS

The isolate of *Saccharomyces cerevisiae* employed in these studies was isolated from packaged Fleischmann's yeast by Mr. J. Hallman in 1950 and had been employed in previous sporulation studies in the Department of Botany, McMaster University. It will be referred to as strain F.J.

Before transfer to the sporulation medium cells were multiplied for 48 hours at 27°C. on the presporulation medium since these conditions were found satisfactory. When a solid presporulation medium was employed the cells were removed with an inoculating needle and suspended in sterilized distilled water. Cells multiplied in liquid media were

centrifuged, washed, and resuspended in sterilized distilled water. This suspension was adjusted to the desired density of cell population with the aid of a haemocytometer. In preparing sporulation cultures, 5 ml. of cell suspension were pipetted into 5 ml. of a sporulation medium of twice the desired concentration in a 16-mm test tube. After thorough mixing, 0.8 ml. of the sporulation culture was pipetted into sterilized shell vials, 15 mm in diameter and 45 mm in length. The depth of the culture in the vials was 5 mm (approximately). Adams (1) reported that depths greater than this prevented sporulation. The vials were incubated at 27°C. for 7 days and then the percentage of cells that had formed asci in a given sporulation culture was estimated by examining 200 cells from each vial. Only asci containing 2, 3 and 4 ascospores were counted as a one-spored ascus might be mistaken for a vegetative cell.

Most solid presporulation media were prepared from commercial dehydrated ingredients. Those frequently employed were:

(a) Basal agar - a synthetic medium of the following composition: 1% dextrose, 0.5% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.01% $FeSO_4$ and 2% agar in distilled water. This medium is the same as Czapek's solution agar however the carbon source and nitrogen source have been changed.

(b) Yeast morphology agar - a synthetic medium developed

by Wickerham (32) and sold commercially by the Difco Corporation. Yeast morphology agar contains 1% dextrose 0.35% $(NH_4)_2SO_4$, 0.15% asparagine, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl and 0.01% $CaCl_2 2H_2O$.

The following vitamins are present in this medium: biotin, 2μg/l, calcium pantothenate, 400μ g/l; folic acid, 2μg/l; inositol, 2000μ g/l; niacin, 400μ g/l; para aminobenzoic acid, 200μ g/l; pyridoxine hydrochloride, 400μ g/l; riboflavin, 200μ g/l; and thiamin hydrochloride, 400μ g/l. Trace elements and three amino acids (histidine, methionine, and tryptophan) are also included in the medium in concentrations that may be obtained from Wickerham's paper.

(c) Vitamin-free-yeast-base agar - prepared by adding 2% agar to the vitamin-free liquid medium developed by Wickerham (32) and sold commercially by the Difco Corporation. This medium, used for vitamin deficiency tests, is a liquid medium of similar composition to yeast morphology agar but contains 0.5% $(NH_4)_2SO_4$ as the nitrogen source and does not contain vitamins.

(d) Czapek's solution agar (Difco).

(e) Lodder's medium - as described in Introduction (page 5)

(f) Dextrose nutrient agar - prepared by adding 5% dextrose and 2% agar to nutrient broth (Difco).

(g) Potato dextrose agar (Difco).

(h) Wort agar (Difco).

(i) Tomato juice agar (Difco).

(j) Tomato juice agar - prepared by adding 2 grams of agar and 10 ml. of distilled water to 90 ml. of filtered commercial tomato juice.

(k) Czapek - Dox agar-prepared by adding 2% agar to Czapek-Dox broth (Difco).

The solid presporulation media were dispensed in 5-ml. quantities to 16-mm test tubes, which were slanted so that no butt was present at the bottom of the tube.

Most vitamin studies were conducted with liquid media to avoid the difficulty of obtaining vitamin free agar. The liquid media employed were;

(a) Basal medium - as described above (page 7) but lacking agar. This medium was prepared as a stock solution of twice the desired concentration.

(b) Vitamin-free-yeast-base-as described above (page 8) but lacking agar.

(c) Dextrose nutrient broth - prepared by adding 5% dextrose to nutrient broth (Difco).

(e) Tomato juice-prepared by adding 0.5 ml. of distilled water to 4.5 ml. of filtered commercial tomato juice.

(f) Czapek-Dox broth (Difco).

When preparing liquid basal presporulation medium containing vitamins, stock vitamin solutions were pipetted into 13-mm test tubes or 2-ounce pharmaceutical bottles and distilled water was added to bring the volume up to 2 ml. After sterilization of this solution, 8.5 mls. of sterilized

stock solution of the basal medium was pipetted aseptically into the vitamin solution.

When inoculating liquid presporulation media, yeast cells from a 48-hr. subculture on the vitamin-free-yeast-base agar, with six vitamins added, were suspended in sterilized distilled water, and washed by centrifuging and resuspending in sterilized distilled water. This suspension was adjusted to the desired cell population density with a haemocytometer. One-half ml. of this suspension was pipetted into 4.5 ml. of the liquid medium giving a total volume of 5 ml. in each pharmaceutical bottle or test tube.

The growth response of the F.J. strain in liquid media containing vitamins was estimated turbidometrically with a simple instrument developed by Richardson (24). Growth was also estimated by examination with the haemocytometer of 0.0001 ml. volumes of the media.

Liquid presporulation cultures were incubated in 2-ounce pharmaceutical bottles 35 x 10 mm at the base and 95 mm high with a neck 10 mm in diameter. These bottles were sloped on the flat side so that a shallow depth of medium (approximately 5 mm) would assure an oxygen supply to the cells.

All media were autoclaved at 15 lbs. pressure for 15 minutes. Glassware, previously cleaned in sulfuric acid-dichromate cleaning solution, was dry-sterilized at 170°C. for 1 hour.

EXPERIMENTAL

(A) THE EFFECT OF CELL NUMBERS ON SPORULATION IN 0.1% SODIUM ACETATE SOLUTION.

Stantial (28) and Adams (1) reported that sporulation varied greatly with the number of cells per ml. of a given sporulation medium, and indicated suitable densities of cell population for obtaining high ascospore yields. Adams (1) also found that the optimum number of cells per ml. of sporulation medium varied with different strains.

Cells harvested from 48 hr. cultures on 5% dextrose nutrient agar + 0.1% yeast extract were placed in 0.1% acetate solution and in distilled water in population densities of 0.5 to 20 million cells per ml. to ascertain a suitable population density for use in experiments with the F.J. strain. The yields of asci present after 7 days are listed in Table 1. The yield of asci was reduced when the population density was above 5 million cells per ml. or below 1 million cells per ml.

Cells transferred to distilled water gave very low yields of asci never exceeding 2%. Since this observation was general during the course of the investigation, the yields of asci in these water controls will not be tabulated.

(B) THE EFFECT ON SPORULATION OF VARYING THE CONCENTRATION OF SODIUM ACETATE IN THE SPORULATION MEDIUM.

A second preliminary experiment, in which the

concentration of sodium acetate in the sporulation medium was varied from 0 to 1.0%, was performed to indicate whether an acetate concentration of the order of that used by Stantial (28) would give satisfactory results with the F.J. strain. Cells were taken from 48 hr. cultures on 5% dextrose nutrient agar + 0.1% yeast extract. The population density of cells was kept constant at 1 million cells per ml. since this seemed appropriate from the results of (A) above. The yields of asci present after seven days are listed in Table II. It is evident that yields of asci were reduced above and below the sodium acetate concentrations of 0.1 to 0.2%.

In view of the results of the preceding experiments the cell population density was maintained at 1 million and the sodium acetate concentration at 0.1%.

(C) THE FORMATION OF ASCI IN ACETATE SOLUTION BY CELLS FROM TWELVE PRESPORULATION MEDIA.

TABLE I

The effect of the cell population density in 0.1% sodium acetate solution on the yield of asci.

Millions of cells per ml. of sporulation medium	Yield of asci after 7 days Per cent
40	0.2*
20	0.3
10	3.6
5	16.4
2	12.4
1	15.9
0.5	5.4

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

The standard error between treatments is ± 2.2.

L.S.D. = 4.5 for P 0.05
= 6.1 for P 0.01

TABLE II

The effect on the yield of asci of the sodium acetate concentration in sporulation medium containing 1 million cells per ml.

Concentration of sodium acetate	Yield of asci after 7 days
Per cent	Per cent
1.00	7.6*
0.60	9.7
0.30	13.8
0.20	19.6
0.15	20.4
0.10	23.1
0.05	11.5
0	1.0

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

The standard error between treatments is ± 2.1.

L.S.D. = 4.4 for P 0.05
5.8 for P 0.01

In initiating nutritional studies the yeast was multiplied on twelve presporulation media. Growth on these media was estimated by an examination of the agar slant 48 hours after inoculation. Cells were transferred from the twelve media to 0.1% sodium acetate solution and sporulation in this medium was determined in seven days. The results, listed in Table III, clearly indicated that the presporulation media varied in their effect on growth and sporulation. The conclusions drawn from these results are as follows:

(i) Czapek's solution agar allowed slight growth and the cells thus provided were incapable of sporulation.

(ii) The addition of yeast extract and peptone to Czapek's solution agar stimulated both growth and sporulation. Fairly large quantities seem to be required for the maximum effect.

(iii) The addition of yeast extract to 5% dextrose nutrient agar stimulated sporulation but did not appear to affect growth. Presumably the yeast extract supplied or supplemented factors essential for sporulation that are lacking, or in low supply, in 5% dextrose nutrient agar. This suggests that the use of 5% dextrose nutrient agar in genetic and taxonomic studies as a presporulation medium would not be fully satisfactory.

(iv) Two biotic media, wort agar and tomato juice agar, gave yields of ascospores comparable to 5% dextrose nutrient agar

TABLE III

Ascospore formation in 0.1% acetate solution by cells from 12 presporulation media.

Presporulation medium	Growth on presporulation medium	Yield of asci after 7 days Per cent
5% Dextrose nutrient agar	+++*	16.2**
5% Dextrose nutrient agar + 0.1% yeast extract	+++	25.8
5% Dextrose nutrient agar + 1% yeast extract	+++	34.3
Czapek's solution agar	+	0
Czapek's solution agar + 0.1% yeast extract	++	3.7
Czapek's solution agar + 1% yeast extract	+++	35.2
Czapek's solution agar + 0.1% peptone	++	2.1
Czapek's solution agar + 1% peptone	+++	22.7
Lodder's medium	+	6.9
Wort agar	+++	31.2
Tomato juice agar	+++	37.5
Potato dextrose agar	++	9.4

* + = slight growth; ++ = moderate growth; +++ = abundant growth.

** Each figure based on an examination of 1000 cells; 200 from each of 5 vials.
The standard error between treatments is ± 0.98.

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

+ 1% yeast extract, whereas a third, potato dextrose agar, did not. Presumably potatoes are deficient in factors that occur in wort, tomato juice, peptone and yeast extract.

(v) Cells multiplied on Czapek's solution agar without added yeast extract did not form ascospores when transferred to acetate solution but with the other synthetic presporulation medium (Lodder's medium) a low yield of asci resulted. Experiments to be described indicate that this was due to the fact that the nitrogen source in Czapek's solution agar is $NaNO_3$ whereas in the other medium it is $(NH_4)_2SO_4$.

(D) THE EFFECT OF VITAMINS ON SPORULATION AND GROWTH.

1. The formation of asci in acetate solution by cells from solid synthetic media.

In an attempt to find a synthetic medium for use as a presporulation medium in the investigation, cells were multiplied on various synthetic media; growth was estimated by an examination of the agar slant 48 hours after inoculation; and cells were transferred to acetate sporulation medium. The six vitamins biotin, pantothenate, thiamin, niacin, inositol and pyridoxine were added to the three media which did not contain vitamins. The vitamins were used in the concentrations employed by Wickerham (32) in certain media he recommends for yeast classification. The other medium, yeast morphology agar, described in Methods and Materials contained three additional vitamins folic acid, p-aminobenzoic acid, and riboflavin.

The yields of asci present in sporulation cultures after 7 days are listed in Table IV. Cells multiplied on yeast morphology agar, vitamin-free-yeast base agar + 6 vitamins, and on basal agar + 6 vitamins formed asci in yields comparable to cells from Czapek's solution agar + 1% yeast extract. These synthetic media probably contained all the factors essential to sporulation which are present in Czapek's solution agar + 1% yeast extract. Since yeast morphology agar contained 3 vitamins in excess of vitamin free yeast base agar + 6 vitamins, evidently these three vitamins were not essential to growth or sporulation of the F.J. strain. Basal agar + 6 vitamins, which is a simple medium, must have contained all the factors essential for growth and sporulation of the F.J. strain, since this medium supported growth and gave high yields of ascospores. It was therefore used in the experiments to follow.

Vitamin-free-yeast-base agar and basal agar did not support growth or sporulation as well as when vitamins were added. The fact that any growth occurred was probably due to growth factor impurities in the agar.

Czapek's solution agar and this medium with 6 vitamins added allowed slight growth and the cells thus provided were incapable of forming asci. Since the only differences between Czapek's solution agar and basal agar are the nitrogen and carbon sources, the most probable explanation

TABLE IV

Ascospore formation in 0.1% acetate solution by cells from synthetic presporulation media.

Presporulation medium	Growth on presporulation medium	Yield of asci after 7 days Per cent
Yeast morphology agar	+++*	28.7**
Vitamin-free-yeast-base agar	++	8.5
Vitamin-free-yeast-base agar + 6 vitamins	+++	27.9
Basal agar	++	18.7
Basal agar + 6 vitamins	+++	29.2
Czapek's solution agar	+	0
Czapek's solution agar + 6 vitamins	+	0
Czapek's solution agar + 1% yeast extract	+++	30.2

* + = slight growth; ++ = moderate growth; +++ = abundant growth.

**Each figure based on an examination of 1000 cells; 200 from each of 5 vials.
The standard error between treatments is ± 0.78.

L.S.D. = 1.7 for P 0.05
2.2 for P 0.01

is that the yeast was incapable of utilizing the $(NO_3)^-$ nitrogen source in the Czapek's medium.

2. The vitamin requirements for growth of the F.J. strain.

(a) Before attempting to assess the effect of individual vitamins on sporulation, the vitamins required for growth of the F.J. strain were determined.

Multiple vitamin deficiencies may be detected by employing Burkholder's (5, 6) procedure of deleting one vitamin at a time from a synthetic medium known to support growth.

Thirteen-mm. test tubes containing the basal medium plus vitamin combinations were inoculated with 0.1 million cells per ml. The tubes were incubated at 27°C. and the turbidity of the yeast in the medium was determined with a simple turbidometer 24, 36, 48 hours and 1 week after inoculation.

The results tabulated in Table V indicate that the F.J. strain required an exogenous source of biotin for growth. The organism also required a supply of pantothenate for growth in the first 48 hrs. At the end of one week however growth in the medium lacking pantothenate was as great as in the medium containing six vitamins. This suggests that the strain under investigation can slowly synthesize pantothenate. Niacin and thiamin were required in the medium for optimum growth in 24 hours but within

TABLE V

Growth of the F.J. strain in basal medium containing various combinations of vitamins.

Substances added to basal medium	Turbidity values: Time after inoculation (days) 0	1	1½	2	7
all 6 vitamins	35*	7	4	3	--
5 vitamins (biotin deleted)	30	29	25	25	24
" (pantothenate ")	35	35	30	33	5
" (inositol ")	30	10	7	3	--
" (niacin ")	30	30	7	3	--
" (pyridoxine ")	30	10	3	3	--
" (thiamin ")	33	27	8	3	--
No vitamins	35	35	32	32	32
1% yeast extract	30	10	4	3	--

*Foot candles of light transmitted through culture; varies inversely as the density of cell population in the culture.

36 hours the yeast overcame its initial need for these two vitamins.

(b) The effect of biotin, pantothenate, niacin, and thiamin on growth was investigated further. Biotin and pantothenate were added to the medium singly and in combination. Also the two vitamins required by the yeast for optimum growth in 24 hours, niacin and thiamin, were added separately and together to the medium containing biotin and pantothenate in the combinations shown in Figure 2.

Two-ounce pharmaceutical bottles containing the basal medium and added vitamins were inoculated with 0.1 million cells per ml. The bottles were incubated at 27°C. and growth in this experiment was estimated by examining with a haemocytometer 0.0001 ml. volumes of the contents of each culture. The results are presented graphically in Figures 1 and 2.

The results obtained for the basal medium alone and with biotin and pantothenate added alone and in combination are illustrated in Figure 1. In the basal medium + biotin and pantothenate growth resulted in a cell population density of 25 million cells per ml. in 2 days. The yeast in the basal medium + biotin attained a cell population density of 25 million cells per ml. only after 9 days, probably due, as suggested previously, to a slow rate of synthesis of pantothenate. Biotin was apparently not

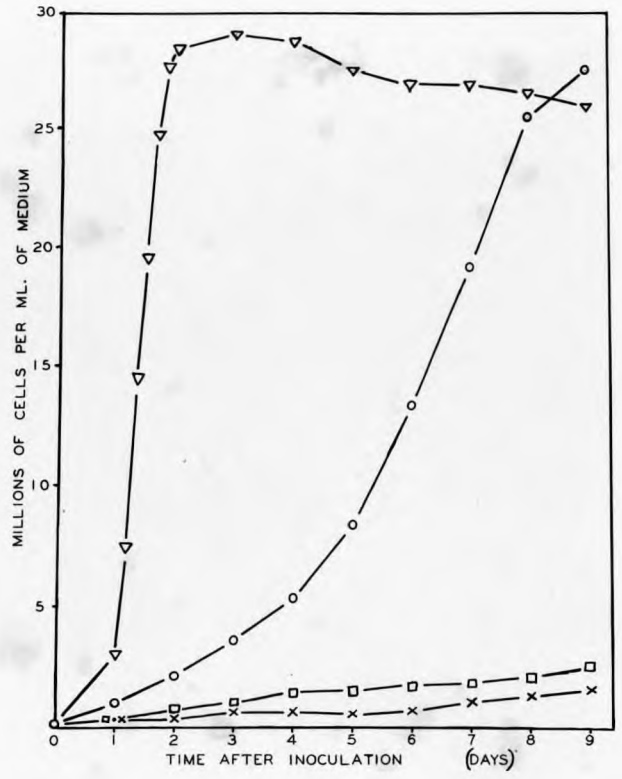

Figure 1. Growth of the F.J. strain in basal medium containing various combinations of vitamins.
× —— × No vitamins. □ —— □ Pantothenate.
○ —— ○ Biotin. ▽ —— ▽ Biotin and pantothenate.

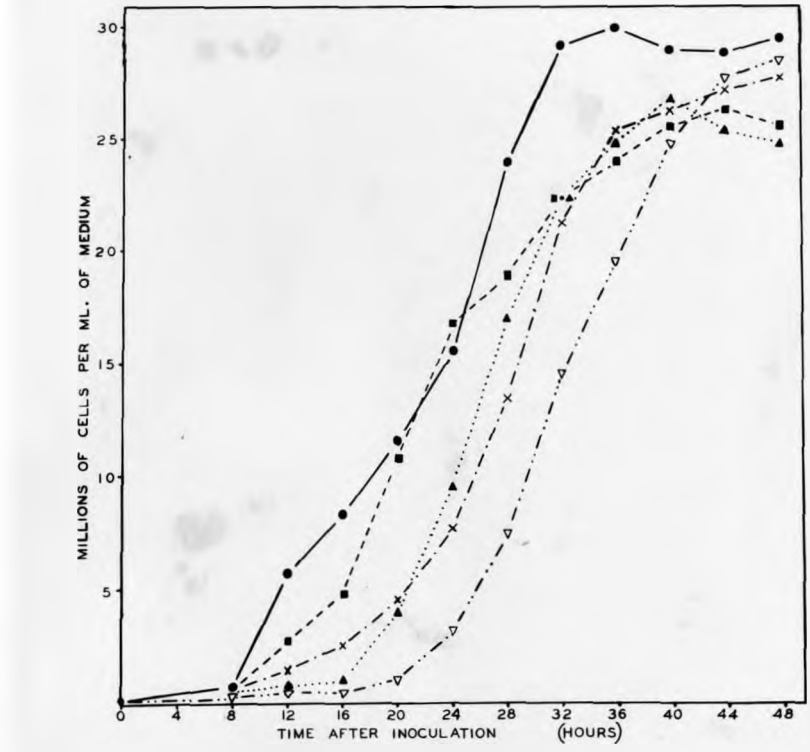

Figure 2. Growth of the F.J. strain in basal medium containing various combinations of vitamins.

- Biotin, and pantothenate.
- Biotin, pantothenate, and niacin.
- Biotin, pantothenate, and thiamin.
- Biotin, pantothenate, niacin, and thiamin.
- Biotin, pantothenate, niacin, thiamin, pyridoxine, and inositol.

synthesized by the F.J. strain, since the basal medium and the basal medium + pantothenate failed to support growth of the yeast over a nine day period.

The growth of the yeast in basal medium containing the remaining combinations of vitamins is illustrated graphically in Figure 2. In addition to confirming the conclusions drawn from the preceding experiment, Figure 2 demonstrates the following points.

(i) The yeast grew slowly with only biotin and pantothenate in the medium. It did not begin to multiply rapidly until at least 24 hours after inoculation.

(ii) The addition of niacin or thiamin to the biotin-pantothenate supplemented medium caused multiplication to begin about 16 hours after inoculation. When both were added active multiplication began about 12 hours after inoculation.

(iii) The addition of pyridoxine and inositol to the medium containing the four vitamins mentioned above caused active multiplication to begin about 12 hours after inoculation.

3. The effect on sporulation of adding vitamins to the presporulation medium.

(a) Stantial (28) found that the addition of Bios II and inositol to a mineral salts-sugar medium greatly increased ascospore formation of cells multiplied on that medium. However, the present studies are to the writer's

knowledge the first time synthetic vitamins have been used in studying the nutrient requirements for yeast sporulation.

The same vitamin combinations were used as in (2 b). However, since cells had to be harvested and transferred to sporulation medium, a sufficiently heavy inoculum had to be added to the presporulation medium to ensure that some multiplication would occur, even in the vitamin deficient basal medium. Preliminary investigations indicated that, when 1 million cells per ml. were added, sufficient growth occurred in the basal medium to provide inoculum for the sporulation medium.

The medium containing the vitamins was inoculated with 1 million cells per ml. Growth, as measured by the cell population density 48 hours after inoculation, was determined with a haemocytometer. The cells were transferred to acetate solution, and sporulation in this medium was determined in seven days. This experiment was repeated three times and the average of the results obtained in these three experiments are presented in Table VI. Conclusions based on this experiment are as follows:

(i) The basal medium failed to support growth and the cells incubated in this medium formed few asci in acetate sporulation medium.

(ii) The addition of biotin to the basal medium

TABLE VI

Ascospore formation in 0.1% acetate solution by cells from basal medium containing various combinations of vitamins.

Substances added to basal medium	Cell population density 48 hrs. after inoculation Million cells per ml.	Yield of asci of all types after 7 days Per cent	Yield of four-spored asci Per cent
None	3.7*	1.4**	0***
Biotin	20.8	25.3	1.0
Pantothenate	3.5	9.9	0.1
Biotin and Pantothenate	28.7	24.5	0.6
Biotin, Pantothenate and Niacin	26.1	36.0	1.2
Biotin, Pantothenate and Thiamin	22.2	47.8	1.0
Biotin, Pantothenate, Niacin and Thiamin	24.0	47.5	0.8
Biotin, Pantothenate, Niacin, Thiamin, Pyridoxine and Inositol	28.8	49.2	11.0
1% yeast extract	44.9	48.1	11.4

The figures shown represent an average taken from three experiments.

*In each experiment the cell population density was determined by examining 0.0001 ml. volumes of medium.

**In each experiment the yield of asci was determined by examining 1000 cells; 200 from each of 5 vials.

The standard error between treatments is ± 1.9.

L.S.D. = 3.6 for P 0.05
4.8 for P 0.01

*** The standard error between treatments is ± 0.14.

L.S.D. = 0.3 for P 0.05
0.4 for P 0.01

increased growth and sporulation considerably.

(iii) The addition of pentothenate to the basal medium caused no marked increase in growth but sporulation was distinctly stimulated.

(iv) B* and P* in combination in the basal medium increased the population density at the end of 48 hours incubation to the maximum level (25 - 30 million cells per ml.)Sporulation of cells from this medium was the same as when biotin was used alone.

(v) The addition of four more vitamins N, T, Py and I to the medium containing B and P did not result in any increase in cell population density 48 hours after inoculation. But yields of asci were increased when B, P, and N were present and further increase occurred when B, P, and T were present. The addition of N to B, P, and T caused no clear increase over B, P, and T. The further addition of Py and I caused no increase in sporulation, but in the column to the right of the table an interesting fact will be noted. Though the numbers of asci were not increased by Py and I, the numbers of four-spored asci showed a marked increase and the numbers of such asci were as great as when yeast extract was employed. This observation is of some practical importance in regard to yeast genetics

*Henceforth in this discussion each vitamin will be denoted by the first letter of its name. Pyridoxine will be denoted as Py.

and taxonomy.

(vi) Yeast extract added to the basal medium in a concentration of 1% resulted in a greater cell population density after 48 hours incubation than did any of the above vitamin combinations. Yields of asci and of four-spored asci were similar to those yields obtained from cells multiplied in the basal medium containing six vitamins. Apparently, the synthetic medium with the six vitamins contained all the factors essential to growth, high yields of asci, and the formation of four-spored asci present in 1% yeast extract.

(b) The effect of the addition of the vitamins Py and I to the presporulation medium on sporulation in acetate solution was investigated further to find whether the stimulation of the formation of four-spored asci was due to only one or to both of these vitamins.

Vitamins were added to the basal medium in the combinations listed in Table VII and the bottles were inoculated with 1 million cells per ml. Growth, as measured by the cell population density 48 hours after inoculation, was determined with a haemocytometer. The cells were transferred to acetate solution, and sporulation was determined in seven days. The results, which are presented in Table VII, indicate that Py and I are both required, in addition to B and P, for high yields of four-spored asci.

TABLE VII

Ascospore formation in 0.1% acetate solution by cells from basal medium containing various combinations of vitamins.

Substances added to basal medium	Cell population density 48 hrs. after inoculation Million cells per ml.	Yield of asci of all types in 7 days Per cent	Yield of four-spored asci Per cent
Biotin and Pantothenate	31.5*	24.4**	0.5***
Biotin, Pantothenate and Pyridoxine	31.3	32.2	1.4
Biotin, Pantothenate and Inositol	25.6	51.0	6.2
Biotin, Pantothenate, Pyridoxine and Inositol	30.1	45.4	8.8
Biotin, Pantothenate, Niacin and Thiamin	27.3	51.6	0.5
Biotin, Pantothenate, Niacin, Thiamin and Pyridoxine	24.8	54.0	2.3
Biotin, Pantothenate, Niacin, Thiamin and Inositol	24.3	54.5	1.9
Biotin, Pantothenate, Niacin, Thiamin, Pyridoxine and Inositol	31.0	51.6	8.3
1% Yeast Extract	44.3	50.2	8.9

*Each figure based on an examination of 0.0001 ml. volumes with a haemocytometer.

**Each figure based on an examination of 1000 cells; 200 from each of 5 vials.
The standard error between treatments is ± 1.67

L.S.D. = 3.4 for P 0.05
4.7 for P 0.01

***The standard error between treatments is ± 0.7

L.S.D. = 1.4 for P 0.05
1.9 for P 0.01

With B and P a moderate yield of asci was obtained. As in the preceding experiment, this was doubled when N an T were added, and the further addition of Py and I did not increase the yield of asci but the yield of four-spored asci was increased greatly. The addition of either Py and I to B, P, N, and T increased the yield of four-spored asci significantly. However, they were equally effective.

The addition of Py and/or I to B and P did not affect the amount of growth during the first 48 hours, but profoundly effected sporulation. Py stimulated sporulation and the formation of four-spored asci moderately. I was more effective. The addition of both Py and I to B and P did not increase sporulation as compared with B, P, and I, but four-spored ascus formation was significantly increased.

On the basis of the results it would be difficult to attribute the simulation of four-spored ascus formation to any particular vitamin. The minimum requirements for high yields of four-spored asci appeared to be B, P, Py and I, although B, P, and I gave a moderate yield.

4. The effect of increasing concentration of biotin in the presporulation medium on growth and sporulation.

Above, the effect on sporulation of the addition of vitamins to the presporulation medium was investigated, however the vitamin concentration was not varied. In an attempt to differentiate between the biotin requirements of

yeast for growth and for sporulation on a quantitative basis, the concentration of biotin in the presporulation medium was varied, and the effect on growth and sporulation was observed.

Biotin was added in varying concentration to the basal medium, containing pantothenate, niacin, thiamin, pyridoxine and inositol. The medium was inoculated with 1 million cells per ml. Growth, as measured by the cell population density 48 hours after inoculation, was determined with a haemocytometer. The cells were transferred to acetate solution, and sporulation in this medium was determined in seven days.

The results are presented graphically in Figures 3, 4 and 5. Each point on the graphs in Figures 3 and 4 is based on an examination of 0.0001 ml. volumes of medium with a haemocytometer. Each point on the graphs in Figures 3 and 5 is based on an examination of 1000 cells, 200 from each of 5 vials.

Figure 3 shows that the cell population density 48 hours after inoculation increased with increasing concentration of biotin up to a concentration of 0.6 μg/l. Further increase in biotin concentration did not affect the cell population density 48 hours after inoculation. Figure 3 also shows that increasing the concentration of biotin in the medium in which yeast cells are grown leads to an increase in sporulation when the cells are transferred to the sporulation medium. However sporulation continued to

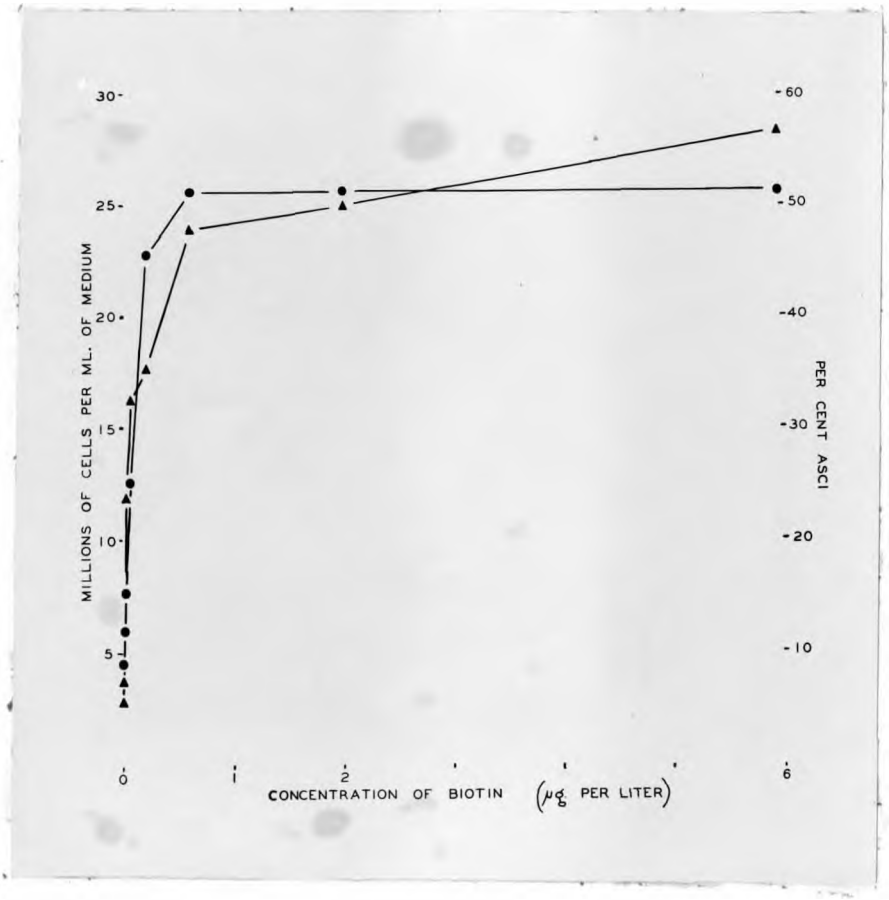

Figure 3. The effect of the concentration of biotin in the presporulation medium on growth and sporulation of the F.J. strain. ● = Growth. ▲ = Sporulation. The standard error for yields of asci is ± 1.9. L.S.D. = 3.8 for P. 0.05, 5.2 for P. 0.01.

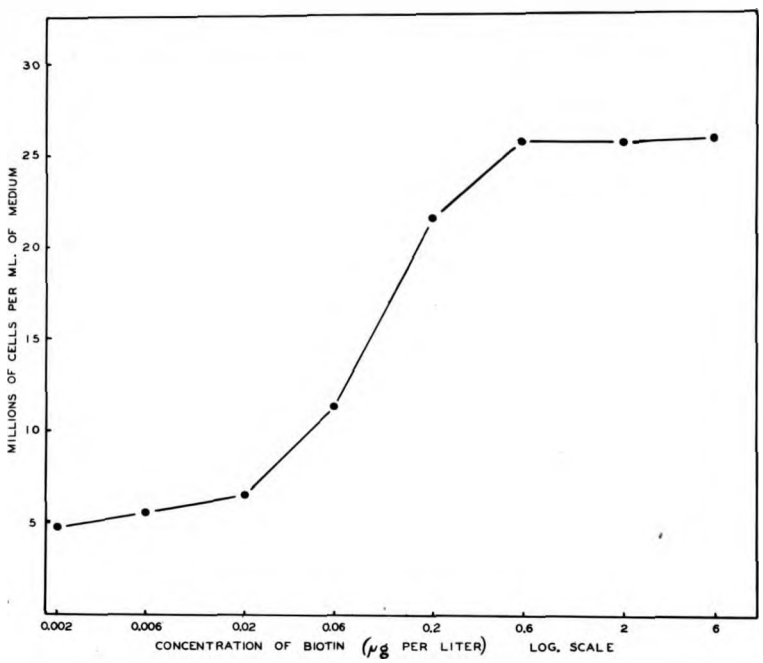

Figure 4. The effect of the concentration of biotin in the presporulation medium on growth of the F.J. strain.

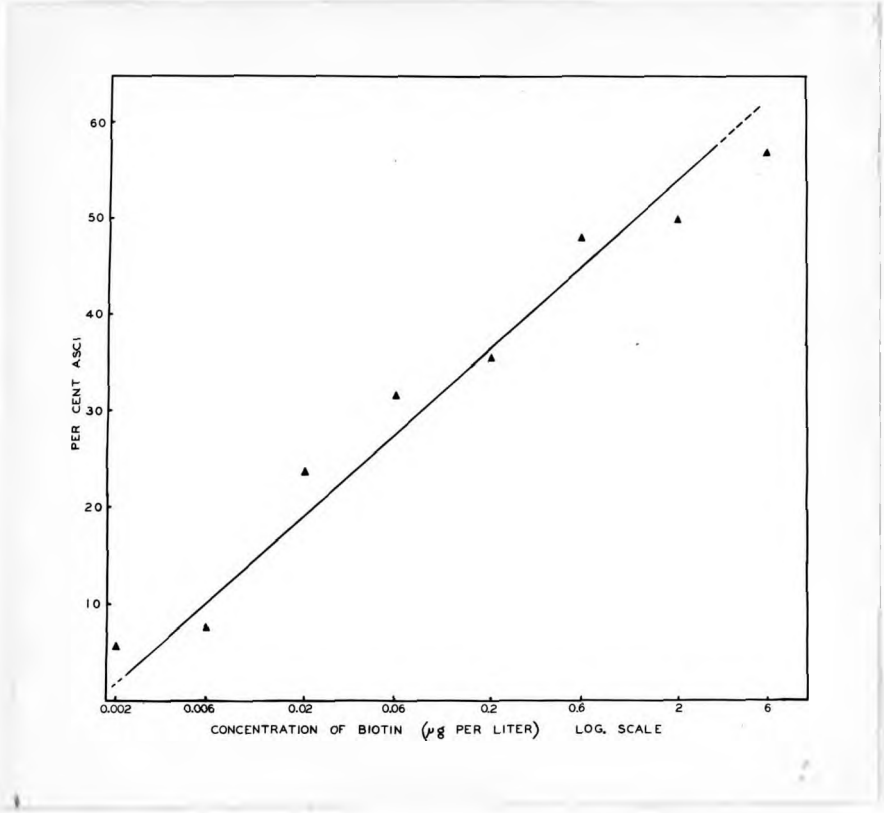

Figure 5. The effect of the concentration of biotin in the presporulation medium on sporulation of the F.J. strain. The standard error between treatments is ± 1.9. L.S.D. = 3.8 for P. 0.05, 5.2 for P. 0.01.

increase above the biotin concentration of 0.6 μg/l. This observation is illustrated very well by a comparison of Figures 4 and 5. It may be concluded that the concentration of biotin required in the presporulation medium for maximum yields of asci is greater than the concentration required for optimum growth in 48 hours.

(E) THE EFFECT OF DILUTING THE PRESPORULATION MEDIUM ON GROWTH AND SPORULATION.

In the two preceding experiments it was noted that cells did not multiply in the basal medium and that these cells formed asci in low yields when placed in acetate solution. These results have raised the question as to whether the failure to produce asci was due to an insufficient supply of vitamins, or to limited cell multiplication in the presporulation medium. The latter possibility is suggested in view of Klebs' (11) generalization that growth, or rather the period of assimilation accompanying growth, is a preliminary step for reproduction.

To investigate this further, cells were multiplied in various dilutions of the basal medium each of which contained the concentrations of the six vitamins used previously. As a control, the same dilutions of the basal medium were used without vitamins. The medium was inoculated with 1 million cells per ml., and incubated at 27°C. for 48 hours. Growth, as measured by the cell population density

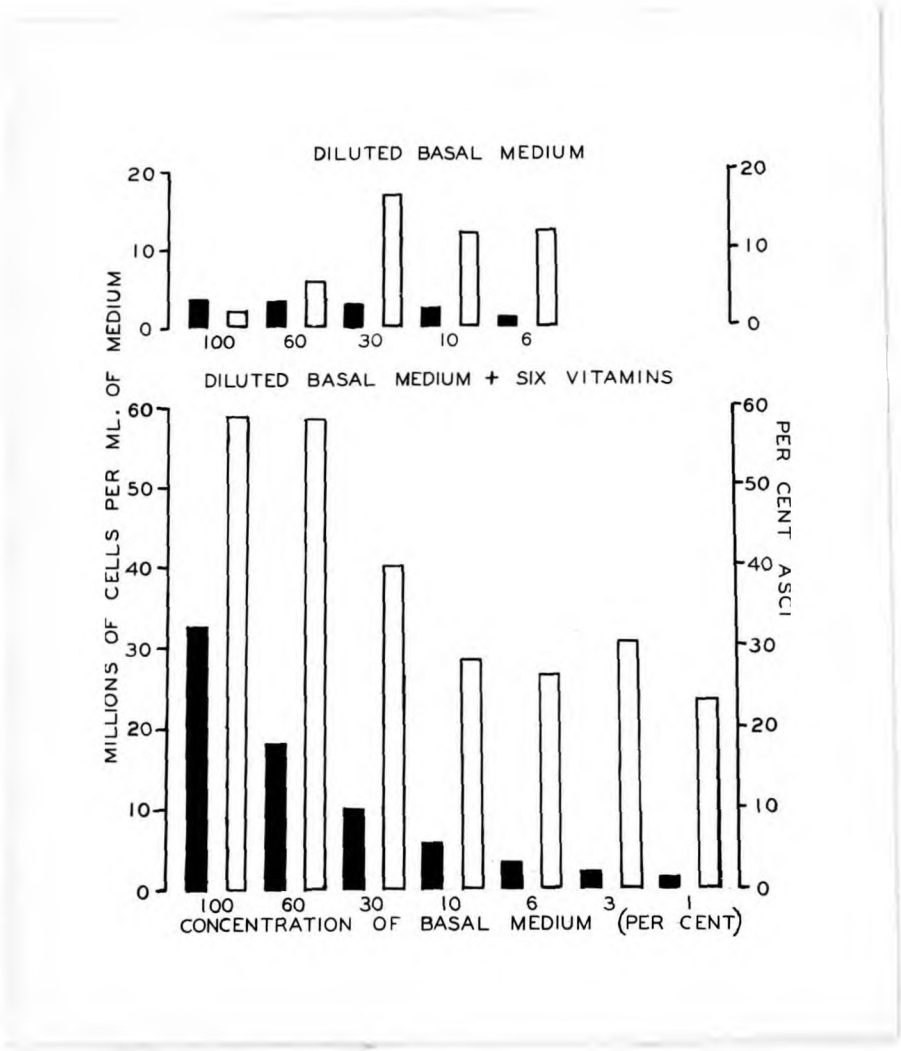

Figure 6. The effect of dilution of the presporulation medium on growth and sporulation. Solid bars = growth. Blank bars = sporulation. The standard error for yields of asci is ± 1.4. L.S.D. = 2.7 for P. 0.05, 3.1 for P. 0.01.

48 hours after inoculation, was determined with a haemocytometer. The cells were transferred to acetate solution and sporulation in this medium was determined in seven days. It was considered that cells would multiply little in the diluted medium containing vitamins and that sporulation of these cells could be compared with cells that had multiplied in a similarly diluted medium but lacking vitamins. Thus an attempt could be made to correlate sporulation with either growth or the presence of vitamins.

The results are presented in Figure 6. Each bar in the graph representing growth is based on an examination of 0.0001 ml. volumes of medium with a haemocytometer. Each bar representing ascospore formation is based on an examination of 1000 cells; 200 from each of 5 vials. In the absence of vitamins, dilution of the presporulation medium resulted in only a slight decrease in growth, although the yields of asci increased with dilution of the presporulation medium. When vitamins were present, dilution of the medium caused a marked decrease in the cell population density 48 hours after inoculation and this was paralleled by a decrease in sporulation when the cells were transferred to sporulation medium. Although the cell population density 48 hours after inoculation in 1% concentration was one tenth that of undiluted medium, decrease in sporulation was only one half. This would suggest that growth in the presporulation medium as measured by the cell population density 48 hours after inoculation was not closely correlated with sporulation of

these cells in sporulation medium.

The yields of asci from cells multiplied in the diluted medium containing vitamins were greater than the yields of asci obtained from cells that had multiplied to the same extent in the diluted medium without vitamins. This would suggest that, the absence of growth in a medium lacking vitamins and the lack of vitamins are both responsible for low yields of asci.

(F) THE SPORULATION OF CELLS IN ACETATE SPORULATION MEDIUM CONTAINING VITAMINS.

It was noted above that cells from the basal medium formed few asci in acetate solution, presumably due to a deficiency of vitamins in the cells. The possibility of supplying the vitamins to these cells in the sporulation medium seemed worthy of investigation.

Cells from 48-hour suboultures in basal medium were transferred to solutions of vitamins of the concentrations used above in the presporulation medium, to 0.1% acetate solution, and to acetate solution containing vitamins. As a control, cells from basal medium + six vitamins were transferred to sporulation media of the same composition.

The yields of asci obtained are presented in Table VIII. Sporulation did not occur when acetate was not present in the sporulation medium, whether or not the cells were multiplied in the presence of vitamins. Cells from the vitamin-free basal medium formed few spores in the acetate

TABLE VIII

The effect of the addition of vitamins to the sporulation medium on ascospore formation.

Composition of sporulation medium	Yield of asci obtained from cells multiplied on basal medium Per cent	Yield of asci obtained from cells multiplied on basal medium + 6 vitamins Per cent
Distilled water	0*	0*
" + biotin	0	0
" + pantothenate	0	0
" + biotin and pantothenate	0	0
" + six vitamins	0	0
0.1% acetate	2.7**	47.6***
" + biotin	7.7	48.0
" + pantothenate	5.9	53.3
" + biotin and pantothenate	8.6	62.2
" + six vitamins	10.4	62.1

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

**The standard error between treatments is ± 1.2.

L.S.D. = 2.6 for P 0.05
3.5 for P 0.01

***The standard error between treatments is ± 1.9.

L.S.D. = 4.0 for P 0.05
5.5 for P 0.01

solution. The yield of asci from the cells was much increased when vitamins were added to the acetate sporulation medium, but it did not exceed 10.4%. Both biotin and pantothenate were stimulating, and the addition of four more vitamins did not cause a significant further increase.

From these results it may be concluded that if cells are starved for vitamins in the presporulation medium, their deficiency can only be partially overcome by adding vitamins to the sporulation medium. This suggests that in addition to acetate and vitamins, a third factor is required for maximum sporulation. Perhaps the cells must be in a suitable physiological state before transfer to the sporulation medium.

Although both biotin and pantothenate stimulated the sporulation of vitamin-starved cells, only pantothenate stimulated sporulation of the cells from basal medium + six vitamins. This suggests that pantothenate may be more directly concerned with the sporulation process, and it is of interest in this connection to note that Novelli and Lipmann (20) found pantothenate to be involved in the oxidation of acetate by yeast.

(G) THE FORMATION OF ASCI IN ACETATE SOLUTION BY CELLS FROM SOLID AND LIQUID PRESPORULATION MEDIA.

The preceding investigations indicated that cells from liquid basal medium + six vitamins gave greater yields of asci in acetate solution than cells from basal agar + six vitamins. This might indicate that liquid media are more suitable as presporulation media with acetate solution as the sporulation medium.

The sporulation of cells multiplied on solid and in liquid media of the same composition was compared in a single experiment. The solid media were inoculated with an inoculating needle. The liquid media were inoculated with 1 million cells per ml. of medium with a suspension of yeast cells in water. The subcultures were incubated at 27°C. for 48 hours. Growth was then determined by an examination of the agar slant. Growth in the liquid media, as measured by the cell population density 48 hours after inoculation, was determined with a haemocytometer. Since the cells from liquid media were always washed before their transfer to acetate sporulation medium, the same procedure was followed with cells from solid media. Sporulation in acetate solution was determined in seven days.

The results are presented in Table IX. In every case the yields of asci obtained from cells multiplied in liquid media were greater than the yields of cells from solid media. Sporulation of the yeast in acetate solution must have been

TABLE XX

Ascospore formation in 0.1% acetate solution by cells from solid and liquid presporulation media.

Presporulation medium	Growth	Yield of asci after 7 days	Presporulation medium	Cell population density 48 hrs. after inoc.	Yield of asci after 7 days
Solid		Per cent	Liquid	million cells per ml.	Per cent
Basal agar + six vitamins	+++*	24.7**	Basal medium + six vitamins	38.7***	48.8**
Vitamin-free-yeast-base agar + six vitamins	+++	24.8	Vitamin-free-yeast-base + 6 vitamins	42.3	30.4
Czapek-Dox agar + 1% yeast extract	+++	24.8	Czapek-Dox broth + 1% yeast	125.4	62.7
5% Dextrose nutrient agar + 1% yeast extract	+++	30.5	5% Dextrose nutrient broth + 1% yeast extract	142.8	56.0
Tomato juice agar	++	28.1	Tomato juice	93.6	69.4

* + = slight growth; ++ = moderate growth; +++ = abundant growth

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

The standard error for yields of asci is ± 1.95.

L.S.D. = 3.8 for P 0.05
5.3 for P 0.01

***Each figure based on an examination of 0.0001 ml. volumes with a haemocytometer.

inhibited by the multiplication on the agar slant.

It is of particular interest to note that there were only small differences between the yields of asci obtained from the solid media, yet the differences between the sporulation of cells from liquid media were great. Apparently there were factors present in three media (Czapek-Dox broth + 1% yeast extract. 5% Dextrose nutrient broth + 1% yeast extract and tomato juice) that were not present in the synthetic media used. Since these factors were present in the corresponding agar also, the agar may have hindered the diffusion of these factors to the cells.

(H) THE EFFECT OF CERTAIN NUTRIENTS ON THE SPORULATION OF STARVED CELLS.

During the course of the investigation the effect of certain nutrients and vitamins on the sporulation of cells starved in different manners was investigated, since such studies might provide an insight into the manner in which they affect sporulation.

1. The effect of yeast extract on the sporulation of cells starved by aeration.

Cells from a slant of Czapek's solution agar - 1% yeast extract were suspended in sterilized distilled water and adjusted with the aid of a haemocytometer to a population density of 2 million cells per ml. of water. This suspension was divided into two parts; one was mixed with an equal volume of 0.2% acetate solution; the other was placed in a

flask in an aeration train. The flow of air through the yeast suspension was regulated at the rate of 30 bubbles per minute by adjusting the filter pump. The cells were aerated for 24 hours and then placed in the sporulation media listed in Table X.

The results presented in Table X indicate that aeration of cells previous to placing them in sodium acetate decreased sporulation. However, the addition of 0.1% yeast extract to the sporulation medium overcame this effect probably by supplying the cells with nutrients or growth factors metabolized during aeration.

It may be considered that yeast cells can use nutrients (in addition to acetate) added to the sporulation medium for the formation of ascospores.

The cells in the sporulation medium containing 0.1% sodium acetate and 1% yeast extract multiplied and sporulation decreased. A decrease in sporulation was noted in (B) above when high concentrations of sodium acetate were used in the sporulation medium. This is in accord with the usual experience when working with S. cerevisiae, that conditions favouring rapid growth do not favour sporulation.

These results may with interest be compared with those of (C) above, in which vitamin-starved cells were added to sporulation medium containing added vitamins. Here the 0.1% yeast extract was apparently able to overcome the effect of aeration on sporulation.

TABLE X

The effect of yeast extract on the sporulation of cells starved by aeration.

	Composition of sporulation medium	Yield of asci in 7 days Per cent
Cells directly from presporulation medium	Distilled water	4.4[x]
	0.1% sodium acetate	28.9
Cells aerated for 24 hours	Distilled water	3.8
	0.1% sodium acetate	18.2
	0.1% sodium acetate + 0.1% yeast extract	30.1
	0.1% sodium acetate + 1% yeast extract	0.1

[x]Each figure based on an examination of 1000 cells 200 from each of 5 vials.

The standard error between treatments is ± 1.6.

L.S.D. = 3.2 for P 0.05
4.3 for P 0.01

2. The effect of yeast extract and peptone on sporulation of cells multiplied on Czapek's solution agar.

It was noted previously that cells multiplied little on Czapek's solution agar and that cells from this agar did not form asci in acetate solution. The reason for this lack of growth was considered to be due to the fact that the yeast could not utilize the $(NO_3)^-$ source of nitrogen of this medium. It was considered that cells inoculated onto this medium were starved for nitrogen.

The possibility of supplying nutrients in the sporulation medium to cells starved by multiplying them on Czapek's solution agar was investigated by placing these cells in acetate solution to which varying concentrations of yeast extract and peptone were added. A difficulty was encountered here since the yeast multiplies little or not at all when Czapek's solution agar is used as the presporulation medium. However, the yeast was found to grow to a limited extent on Czapek's solution agar + 0.03% peptone but did not sporulate when transferred to sporulation medium. Using Czapek's solution agar + 0.03% peptone as the presporulation medium, cells were transferred to 0.1% acetate solution containing varying concentrations of yeast extract and peptone. As a control, cells were also transferred to vials with the same concentrations of yeast extract and peptone but lacking acetate.

The results of this experiment are presented graphically in Figures 7 and 8. Each point on the graphs is based on an examination of 1000 cells, 200 from each of 5 vials. Yeast extract stimulated sporulation in the absence of acetate. Greatest yields of asci were obtained when low concentrations of yeast extract + 0.1% acetate were used as sporulation media but sporulation decreased when higher concentrations of yeast extract were employed.

Similar results were obtained with peptone. Here, however the greatest sporulation occurred at a higher concentration than with yeast extract. This would suggest that the stimulating factor (or factors) is more abundant in yeast extract.

3. The effect of various nitrogen sources and vitamins on sporulation.

Above it was noted that cells multiplied in Czapek's solution agar + 0.03% peptone were found to form asci in acetate solution when low concentrations of yeast extract and peptone were added to the acetate sporulation medium. Yeast extract and peptone are complex substances containing many amino acids and vitamins (26). To ascertain the nature of the substances responsible for this stimulation various nitrogen sources were used alone and in combination with acetate solution, and with acetate solution supplemented with vitamins as sporulation media. Before these substances were added to sporulation media, they were tested as nitrogen

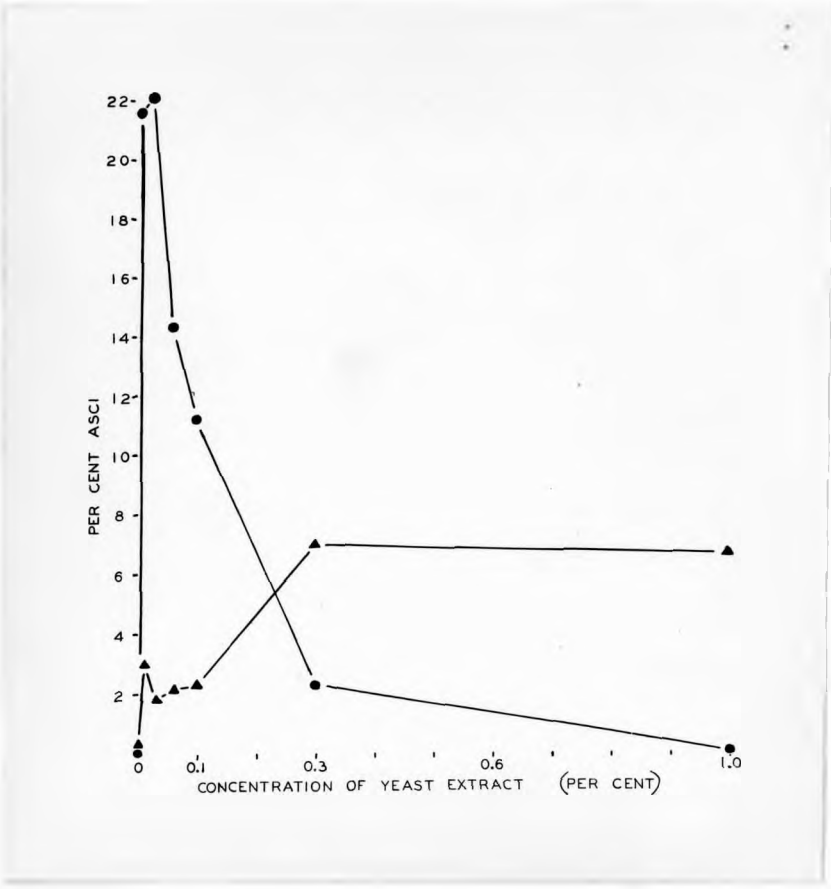

Figure 7. The effect of yeast extract in the sporulation medium on the sporulation of cells from Czapek's solution agar + 0.03% peptone. ▲ = Yeast extract. ● = Yeast extract + 0.1% sodium acetate. The standard error between treatments is ± 0.9. L.S.D. = 1.8 for P. 0.05, 2.4 for P. 0.01.

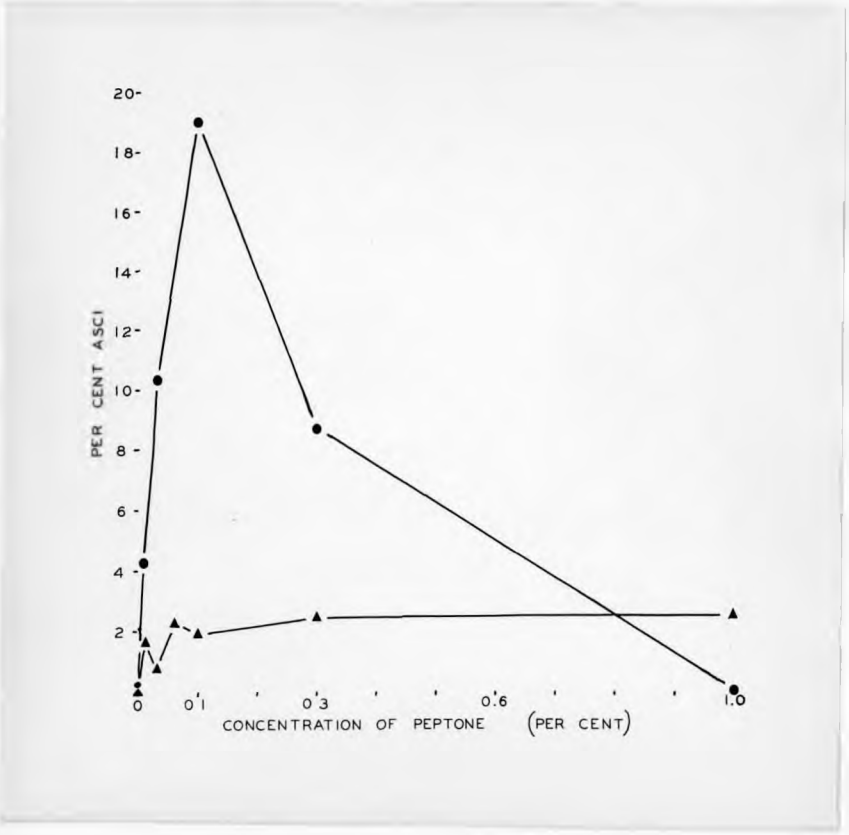

Figure 8. The effect of peptone in the sporulation medium on the sporulation of cells from Czapek's solution agar + 0.03% peptone. ▲ = Peptone. ● = Peptone + 0.1% sodium acetate. The standard error between treatments is + 0.8. L.S.D. = 1.7 for P. 0.05, 2.3 for P. 0.01.

sources in the presporulation medium.

(a) The effect on sporulation of the nitrogen source in the presporulation media.

Inorganic salts, amino acids, asparagine, glucosamine, yeast extract and peptone were added to the liquid basal medium in concentration of 0.5% in lieu of the $(NH_4)_2SO_4$ nitrogen source of this medium. The six vitamins used previously were also added. Each medium was inoculated with 0.1 million cells per ml., and incubated at 27°C. for 48 hours. Growth, as measured by the cell population density 48 hours after inoculation, was determined with a haemocytometer. The cells were then transferred to acetate solution, and sporulation in this medium was determined in seven days.

The results are listed in Table XI. Conclusions based on these results are as follows:

(i) $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, dl-alanine, l-arginine, asparagine, dl-aspartic acid, d-glucosamine, l-leucine, dl-methionine, peptone and yeast extract when used as nitrogen sources supported growth which sporulated from 35 to 50% in acetate solution. The cell population density 48 hours after inoculation varied from 12 to 56 million cells per ml.

(ii) l-tyrosine, and dl-tryptophan supported growth yet cells from these media failed to give high yields of asci in acetate solution. In comparison l-arginine and NH_4Cl supported less growth, yet the cells gave greater yields

TABLE XI

Ascospore formation by cells from basal presporulation medium containing different nitrogen sources.

Nitrogen Source	Cell population density 48 hours after inoculation million cells per ml.	Yield of asci after 7 days Per cent
$(NH_4)_2SO_4$	21*	51.8**
NH_4NO_3	24	37.4
$NaNO_3$	no growth	-
$(NH_4)_2CO_3$	no growth	-
NH_4Cl	14	37.3
dl-alanine	28	41.1
l-arginine	13	34.7
asparagine	40	45.5
dl-aspartic	28	44.6
glycine	no growth	-
d-glucosamine	18	35.1
d-glutamic	no growth	-
l-histidine	no growth	-
l-leucine	26	48.7
dl-methionine	31	44.9
l-tyrosine	25	12.4
dl-tryptophan	25	3.0
peptone	56	47.9
yeast extract	49	49.8

*Each figure based on an examination of 0.0001 ml. volumes with a haemocytometer.
**Each figure based on an examination of 1000 cells; 200 from each of 5 vials.
The standard error between treatments is ± 2.4.

L.S.D. = 4.9 for P 0.05
6.7 for P 0.05

of asci.

(iii) $NaNO_3$, $(NH_4)_2CO_3$, glycine, d-glutamic acid, and l-histidine failed to support growth of the F.J. strain.

(iv) High cell population densities 48 hours after inoculation are not essential to sporulation. For example, peptone supported more growth in the presporulation medium than did $(NH_4)_2SO_4$, yet the cells gave comparable yields of asci in acetate solution.

(b) The effect of various nitrogen sources and vitamins on the sporulation of nitrogen-starved cells.

Cells were multiplied on the basal medium with the nitrogen source deleted but containing six vitamins. These cells were transferred to sporulation medium containing (1) a nitrogen source used above, (2) the nitrogen source + 0.1% sodium acetate, (3) the nitrogen source + 0.1% sodium acetate + six vitamins.

The results listed in Table XII indicate that many nitrogen sources stimulated sporulation when used in combination with 0.1% sodium acetate. The nitrogen sources were not effective when used alone, with the exception of yeast extract and peptone, which were slightly effective. The addition of vitamins to the acetate-nitrogen source sporulation medium increased sporulation in some cases.

$(NH_4)_2SO_4$, dl-alanine, asparagine, l-leucine, peptone and yeast extract gave the largest yields of asci when used with acetate. Ammonium acetate in concentration of 0.01% was also very effective.

TABLE XII

The sporulation of nitrogen starved cells in sporulation media containing various nitrogen sources and vitamins.

Nitrogen source	Yield of asci after 7 days in medium of following composition		
	Nitrogen source alone	Nitrogen source + 0.1% sodium acetate	Nitrogen source + 0.1% sodium acetate + six vitamins
None	0*	0*	0*
0.01% NH_4 acetate	10.5	-	14.8
0.1% NH_4 acetate	0	-	0
0.01% $(NH_4)_2SO_4$	0	2.5	11.1
0.1% $(NH_4)_2SO_4$	0	0	0
dl- alanine	0	20.5	20.8
l - arginine	0	0	0
asparagine	0	11.8	12.5
dl - aspartic	0	2.8	0
d - glucosamine	0	3.3	1.4
d - glutamic	0	0	0.1
glycine	0	6.9	4.0
l - histidine	0	0	0
l - leucine	0	9.1	9.4
dl - methionine	0	2.0	0.6
l - tyrosine	0	4.5	8.1
dl - tryptophan	0	5.0	3.4
yeast extract	1.6	16.6	20.7
peptone	0.3	16.8	19.0

*Based on an examination of 1000 cells; 200 from each of 5 vials.
The standard error between treatments is ± 1.03.

L.S.D. = 2.6 for P 0.05
3.4 for P 0.01

When used with acetate l-arginine d-glutamic acid l-histidine were not effective in stimulating sporulation. However, l-arginine was found to be utilized by the F.J. strain in the presporulation medium in the preceding experiment. Glycine stimulated sporulation in the sporulation medium when used with acetate. However, the yeast evidently did not utilize it as a nitrogen source in the presporulation medium.

It may be concluded that nitrogen-starved cells cannot adequately overcome this lack when nitrogen sources are supplied to them in the sporulation medium to allow them to give high yields of asci. The further addition of vitamins to the sporulation medium does not allow them to give high yields of asci. In a preceding experiment it was shown that vitamin-starved cells supplied with vitamins in the sporulation medium did not provide high yields of asci.

DISCUSSION

A The stimulation of sporulation by acetate.

It is a recognized fact that the nutrient conditions favouring vegetative growth may be quite different from those favouring reproduction. For example, Klebs (10) reported that Saprolegnia mixta tended to grow vegetatively when supplied with abundant food regularly renewed. The organism formed oospores only when transferred to a solid substrate of low nutritive value.

Similar behaviour is found with yeast, for ascospores

are rarely formed by well nourished, actively budding cells. However, when cells of baker's yeast are transferred to a medium of low nutritive value, such as the surface of plaster blocks, or dilute solutions of sugar or acetate, abundant ascospores frequently form.

That the acetate is assimulated is supported by the results of previous work by the author, in which 1,000,000 cells were suspended per ml. of 0.1% sodium acetate solution. The amount of acetate diminished to at least one-half in 48 hours. This result was obtained with the lanthanum nitrate test.

The nature of the stimulation of ascospore formation in yeast resulting from the transfer of yeast cells from a medium rich in nutrients to a dilute solution of acetate is not known. Presumably the more frugal nutrition alters the metabolism in such a manner that instead of buds, ascospores are formed. The purpose of the present study was to compare the effect of certain nutrient factors, particularly micronutrients, on the two processes with the hope of shedding some light on the difference between them.

B <u>The vitamin requirements for growth and for sporulation.</u>

The F.J. strain requires biotin and pantothenate in addition to the usual mineral salts and a source of energy (glucose) for growth in 48 hours. The addition of four more vitamins (niacin, thiamin, pyridoxine, and inositol) in various combinations to the basal medium containing biotin

and pantothenate increased not only the yields of asci from cells multiplied in this medium, but also the yields of four-spored asci. This indicates that the yeast requires more vitamins for maximum sporulation than for growth.

It was noted above that increasing the biotin concentration, up to a concentration of 0.6 μg per liter in the presporulation medium increased growth, as measured by the cell population density 48 hours after inoculation. Sporulation of cells multiplied in basal medium also increased with the biotin concentration of the medium but no limiting concentration was found as in the case of growth. The yeast appears to require a greater concentration of biotin for maximum sporulation than for maximum growth. But since, in this experiment, sporulation occurred in a medium lacking vitamins, a greater supply may need to be carried in the transferred cells to ensure high sporulation.

C <u>The relationship of the extent of cell multiplication in the presporulation medium to subsequent ascospore formation.</u>

The effect of certain necessary vitamins on growth and on sporulation is rather difficult to separate since these vitamins must be present for growth before the effect on sporulation can be observed. The question arises as to whether the failure to produce asci by cells multiplied in a vitamin deficient medium is due to an insufficient supply of vitamins or due to some physiological effect related to the limited cell multiplication. Some light is shed on this

problem by the experiment designed to ascertain whether cells showing limited multiplication in a diluted presporulation medium containing vitamins would sporulate as well as similarly inactive cells from an equally diluted medium lacking vitamins. It was noted that the amount of growth in the presporulation media was correlated with subsequent sporulation in sporulation medium. However, sporulation is greater when cells were multiplied to the same extent in a medium containing essential vitamins. Hence two factors were observed here; (1) a reduction in sporulation related to limited cell multiplication in the presporulation medium; (2) a reduction in sporulation due to the absence of vitamins.

D Proposed explanations for the difference between the vitamin requirements for growth and for sporulation.

(1) The role of different enzyme systems during growth and sporulation.

Since the addition of niacin, thiamin, pyridoxine and inositol are not required for maximum growth in 48 hours, it is probable that these four vitamins can be synthesized by the F.J. strain. The addition of an exogenous supply of these vitamins to the presporulation medium probably allows the yeast cells to accumulate a greater reserve of these vitamins during growth. Although this greater reserve does not affect growth, sporulation in these cells upon transfer to the sporulation medium is

greatly enhanced. The particular enzymes with which these vitamins are thought to be associated may be of greater importance in the metabolism during sexual reproduction than in metabolism during growth.

The effect of pyridoxine and inositol on the production of four-spored asci is very interesting. The supply of pyridoxine and inositol may affect some enzyme system which is involved in formation of spores about the nuclei in the ascus after meiotic division.

The fact that the yeast requires a greater supply of biotin for maximum sporulation than for maximum growth also suggests that the enzyme (or enzymes) for which biotin is a coenzyme is of greater importance in the metabolism during sporulation than during growth. However, there may be unknown reactions involved in the formation of asci which require a biotin coenzyme.

The increase in sporulation caused by the addition of pantothenate to the acetate sporulation medium is another example of the emphasis on particular enzyme systems during reproduction. Since the yeast cannot synthesize biotin, it would seem probable that biotin would be the most effective in increasing yields of asci. This is not the case, however, for the addition of biotin to the sporulation medium does not increase the yield of asci whereas pantothenate does. Since the cells were multiplied in a presporulation medium containing biotin and pantothenate,

it is probable that the cells had a sufficient supply of these vitamins for growth. The further supply of pantothenate in the acetate sporulation medium favoured sexual reproduction in some manner.

Lipmann et al (16) reported the participation of the pantothenate derivative coenzyme A (Co A) in enzymatic acetylation, and more recently Novelli and Lipmann (20) reported that yeast containing a high concentration of Co A respired acetate faster than a yeast containing a lower concentration of this coenzyme. It seems not unreasonable to postulate that the pantothenate supplied to the yeast cells in the sporulation medium favoured the oxidation of acetate, resulting in increased sporulation. There may be, however, other enzyme systems which are affected by Co A that are involved in the formation of asci.

(2) The effect of vitamins on the presporulation experience.

The supply of niacin, thiamin, pyridoxine, and inositol to the basal medium containing biotin, and pantothenate increased ascospore formation in the sporulation medium. It is not unreasonable to postulate that nutrients are stored during growth in the presporulation medium and that sporulation may depend on these stored nutrients. It is possible that this activity is dependent on enzymes that require vitamin coenzymes. In the absence of niacin, thiamin, pyridoxine, and inositol the yeast can synthesize enough of these vitamins for growth in 48 hours

but the storing of nutrients during growth may be limited because of the lower supply of these vitamins.

E The significance of reserve material in sporulation.

There is much evidence to support the view that sporulation is conditioned by the composition of the presporulation medium. It seems probable that one of the factors affecting this conditioning is the vitamin content of that medium. Another possible factor is the amount of nutrients stored during growth on this medium, dependent on the period of assimilation accompanying growth.

The following experimental evidence may be considered as supporting this view.

(1) Cells multiplied in a vitamin deficient medium formed few asci in acetate sporulation medium. When vitamins were added to the acetate an increase in sporulation was noted but yields were low (10.4%). This suggests that for high yields of asci a third factor is required. This factor may be nutrient reserve in the cells. The cells lacking vitamins were unable to store nutrients because of the limited metabolic processes. Evidence that an effect of this nature is probable is given by the work of Williams et al (33) who reported that pantothenate favours the accumulation of glycogen by yeast.

(2) Cells aerated for 24 hours probably metabolized some of the reserve material present in the cells. These aerated cells produced a comparatively low yield of asci in

acetate solution probably because the nutrients stored during the growth in the presporulation medium were metabolized and were not available for metabolism during sporulation. The acetate sporulation medium evidently did not supply the cells with adequate nutrients. Yeast extract, when added to the sporulation medium, overcame the effect of aeration on sporulation probably because it contains many vitamins, amino acids, and some sugar (trehalose).

(3) Cells multiplied on Czapek's solution agar formed asci only when low concentrations of yeast extract and peptone were added to the sporulation medium. The studies of Neilson (19) and the more recent work of Thorne (30) indicates that old yeast cells excrete nitrogen that may be utilized to form many more cells having a lower protein content when transferred to a medium with an unavailable source of nitrogen. Probably this occurred when cells were multiplied on Czapek's solution agar and the cells obtained in this manner did not form asci in acetate sporulation medium. When yeast extract and peptone were added in low concentration to the sporulation medium, the cells may have obtained nitrogen from these substances to proceed with the formation of asci.

(4) Cells multiplied in basal medium with no nitrogen source were probably of low protein content also. Amino acids and other nitrogen sources in the sporulation medium varied in their effect on sporulation. Since some nitrogen sources were as effective as yeast extract and peptone it

seems probable that their function was to supply a source of nitrogen to the starved cells. The work of Thorne (29) indicated that the sole function of amino acids in yeast nutrition was to provide a source of nitrogen. Some sources of nitrogen used in the concentration of 0.1% were probably more effective than others in supplying NH_2 to the cells. When vitamins were added to the acetate sporulation medium containing a nitrogen source there were significant increases in sporulation in some cases. Perhaps one of the vitamins is a coenzyme in an enzyme system in the cell which is responsible for the removal or utilization of the NH_2 of the nitrogen source.

F Other nutritional factors affecting sporulation.

Sporulation of cells multiplied in basal medium with various nitrogen sources varied greatly. There is some difference in the effect of certain nitrogen sources in the sporulation medium and in the presporulation medium. This may indicate some difference in the nutritional role played by these nitrogen sources in these two media. However it must be emphasized that the nitrogen sources were used in one concentration in each medium. The concentration used may not have been the optimum for every nitrogen source. It must also be stated that the effect of nitrogen sources in the sporulation medium was tried only on the sporulation of abnormal nitrogen-starved cells.

Certainly not all the nutritional factors responsible

for high yields of asci have been found in this investigation. Cells multiplied in tomato juice, for example, formed very high yields of asci. This factor (s) may be some vitamin (s) or other nutrient (s) present in an optimum concentration. There is evidently a need for research to find this factor.

G The effect on solid and liquid presporulation media on sporulation.

When solid and liquid presporulation media of the same composition are employed, cells multiplied on the former form fewer asci in acetate solution than cells from the latter. This suggests some form of inhibition by the agar. This inhibition may be due to a slow rate of diffusion of certain nutrients through the agar to the cells on the surface of the agar slant or there may be substances in the agar that inhibit sporulation. The number of cells produced on a 5 ml. agar slant is approximately 5 times as great as the number produced in 5 ml. of a liquid medium of the same composition. The effect then seems not correlated with growth. Adams (1) obtained similar yields of asci from solid and liquid presporulation media. The difference noted here may be due to the use of different strains, or to the fact that Adams used a solid sporulation medium.

H Practical Considerations

Adams (1) described a convenient method of obtaining

ascospores from baker's yeast by using a solid sporulation medium. The writer in previous work was impressed by the simplicity and efficiency of Adams' method.

The present work adds further to our knowledge of the effect of environment on ascospore formation in yeast and increases our control of the process. It offers an explanation in more precise terms of why certain pre-sporulation media are more effective than others. Particularly the stimulation of the formation of four-spored asci by pyridoxine and inositol is of importance in research on yeast genetics since a supply of four-spored asci is essential for crossings, and it offers an inviting opportunity for research on the physiology of sexual reproduction.

Pomper (21) states that Adams' sporulation agar (1) might be of value in the study of the inheritance of adaptive enzymes in yeast since it allows the experimenter to control rigidly the substances to which the yeast is expressed during sporulation. As a result of the present studies a completely synthetic presporulation medium can be employed as well, so that in most instances the cells of the strain under study could be obtained in good condition for sporulation by growing them in a nutritional environment that can be controlled.

SUMMARY

The F.J. strain gave high yields of ascospores when

1 million cells per ml. of 0.1% sodium acetate solution were employed.

Sporulation appeared to depend upon the composition of the presporulation medium.

The F.J. strain was found to require biotin and pantothenate added to the basal medium for growth. Niacin and thiamin were also required for maximum growth in 24 hours.

The vitamin content of the presporulation medium largely determined the yields of asci obtained in acetate biotin and pantothenate gave moderate yields, but niacin and thiamin in addition were required for maximum yields of asci. Pyridoxine and inositol were required also for high yields of four-spored asci.

The concentration of biotin required for optimum growth was lower than the concentration required for the greatest yield of asci.

Sporulation of vitamin-starved cells was increased to 10% by the addition of biotin and pantothenate to the sporulation medium.

Sporulation of cells multiplied in basal medium + 6 vitamins was increased by the addition of pantothenate to the sporulation medium.

The extent of growth in the presporulation medium appears to affect sporulation upon transfer to the sporulation medium.

Cells multiplied in liquid media form greater yields of asci in acetate sporulation medium than cells multiplied on solid media of the same composition.

Ascospore formation by cells starved by aeration and by multiplying them on Czapek's solution agar was stimulated by the addition to the acetate sporulation medium of low concentrations of yeast extract and yeast extract or peptone respectively.

Cells multiplied in basal medium with no nitrogen source formed asci only when 0.1% solutions of nitrogen sources were used in the sporulation medium in addition to the acetate. Vitamins added to the acetate-nitrogen source sporulation medium increased ascospore formation by these cells in some cases.

The sporulation of cells also appeared to depend greatly on the nitrogen source used in the basal medium + 6 vitamins.

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