NUTRITIONAL CONTROL OF ASCOSPOROGENESIS IN AN APOMICTIC STRAIN OF SACCHAROMYCES CEREVISIAE

By

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ABSTRACT

An unusual strain of *Saccharomyces cerevisiae* that produces two-spored asci under conditions in which typical strains produce three and four-spored asci was investigated. It was possible to restore "normal" three and four-spored ascus production in this strain by manipulating the nutritional environment. The effects of nutritional factors supplied during the presporulation and sporulation phases were studied. The change in the proportion of three and four-spored asci was the major parameter employed to quantitate the effect of these factors on ascosporogenesis. Using the Giemsa nuclear stain, germination techniques, and genetic analysis, the number of spores per ascus was related to the manner of nuclear division in developing asci.

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INTRODUCTION

Life Cycle of Saccharomyces cerevisiae

With genetical and cytological evidence, Winge (1935) and Winge and Laustsen (1937, 1939) demonstrated the alternation of haploid and diploid generations in the life cycle of *Saccharomyces* yeasts. When they isolated and cultivated the four spores from a single ascus, they obtained evidence of gene segregation for macromorphological characters. They concluded that segregation by reductional nuclear division had preceded ascospore formation, and that the cells of the cultures derived from the single spores must be haploid. Furthermore, they showed that during or shortly after ascospore germination, diploidization could occur through conjugation between haploid cells followed by nuclear fusion. Like the haploid cells, the diploid zygotes so formed could multiply asexually by budding. *Saccharomyces* yeasts are diploid during most of their life cycle. Lindegren and co-workers (1949) confirmed the findings of Winge and Laustsen. But in addition, they established the existence of a mating system in *Saccharomyces cerevisiae*. When four single spores from one ascus were cultivated, four non-sporogenic haploid colonies developed, two of which were of one mating type (designated *a*), and the other two were of the opposite mating type (designated *α*). Evidence for this particular mating system stemmed from the consistent observation that haploid cells of one mating type could fuse only with haploid cells of the other mating type. Diploid cells remained diploid under conditions

favouring vegetative growth, but when transferred to a medium favouring sporulation, they differentiated into asci containing four haploid ascospores, two of which were a and two of α mating type.

The life cycle of a typical four-spored *Saccharomyces* yeast is shown in Figure 1.

Since the time when sporulation in *Saccharomyces* was first recognized as a sexual process, much attention has been given to the mating behaviour of cells from germinated ascospores (Crandall and Egel, 1977) and strains of *Saccharomyces cerevisiae* have been classified as heterothallic or homothallic with respect to mating capacity.

In the heterothallic strains, clones derived from single ascospores consist of stable haploid cells displaying either a or α mating type. Daughter cells derived from single haploid ascospores can continue to divide equationally by budding in growth medium, but they can never undergo meiotic division in sporulation medium (Lindegren, 1949). Diploidization occurs when haploid cells originating from ascospores of opposite mating type are allowed to fuse. Sometimes rare fusions occur between daughter cells originating from a single haploid ascospore (Lindegren, 1949). In these instances, diploid strains are formed which are homozygous for all loci including mating type. These a/a or α/α diploid cells are capable of mitotic nuclear division in the growth medium, but are incapable of meiotic nuclear division in the sporulation medium (Roth and Lusnak, 1970), and therefore produce no ascospores, although some rare exceptions have been reported (Kozhina, 1978). Sporulation competent tetraploids can

Figure 1: Life cycle of Saccharomyces showing alternation of haploid with diploid generations (after Grewal, 1972).

A, B, C: Budding of a vegetative cell with mitotic division of the diploid nucleus

D : Four haploid nuclei resulting from meiotic division of the diploid nucleus

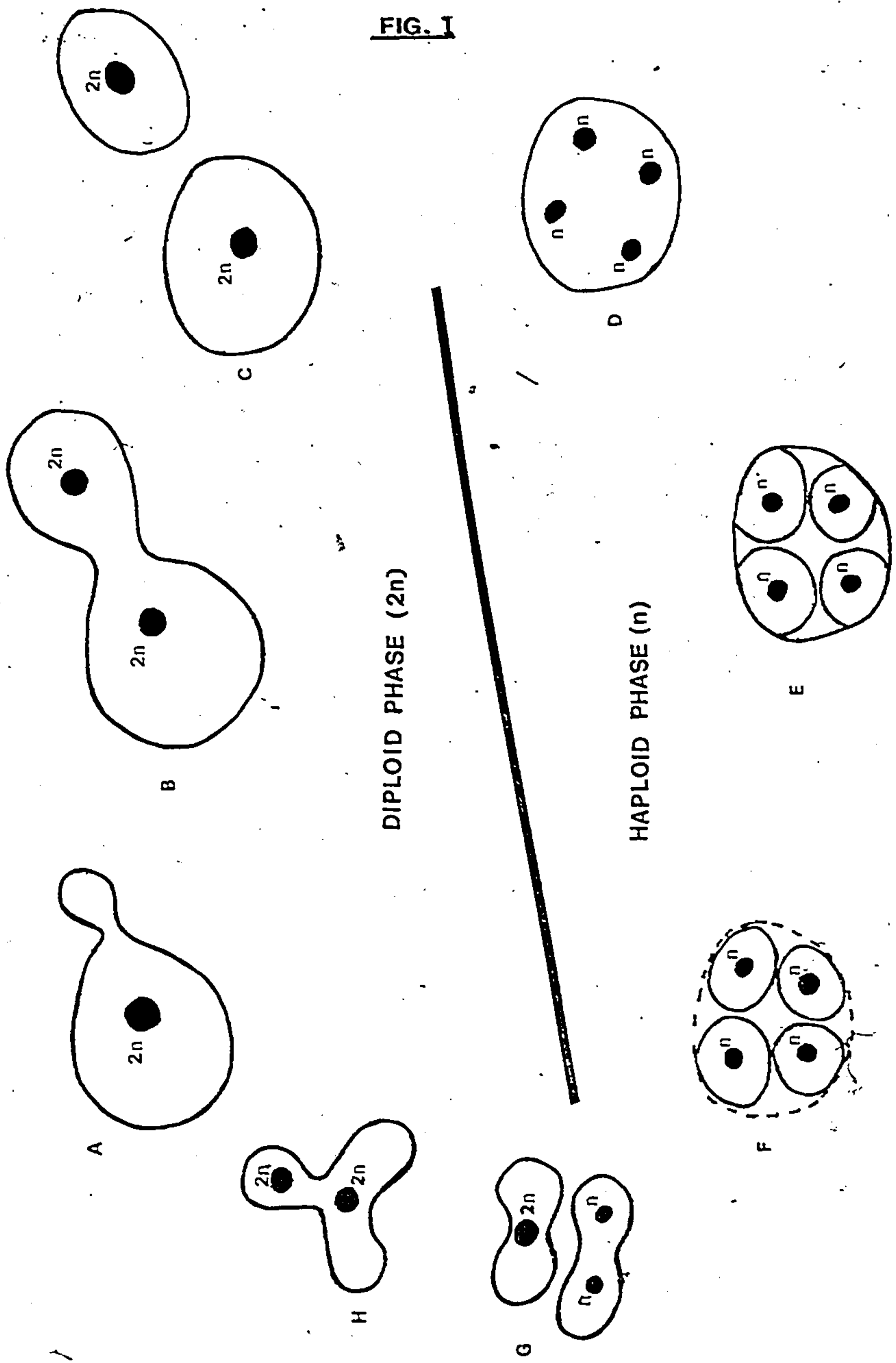
E : Four haploid spores in an ascus, two of which are a mating type and two α mating type

F : Ascus after a few hours in germination medium; spores swell and the ascus wall degenerates (- - - - -)

G : Plasmogamy and karyogamy between spores of opposite mating type

H : Mitotic division of diploid nucleus and production of first bud

FIG. I



be synthesised by pairing diploid strains of opposite mating type. Reduction division then occurs during sporulation and tetranucleate asci form, but the nuclei are diploid.

Homothallic strains differ from heterothallic strains, in that diploidization can occur through fusions between haploid daughter cells of opposite mating type, both descendants of the same haploid ascospore. In Saccharomyces, this phenomenon is controlled by a set of dominant genes designated HO, HM_a, HM_α (Winge and Roberts, 1949; Hawthorne, 1963; Takano and Oshima, 1969, 1970; Hicks and Herskowitz, 1976). The genes for homothallism exert their control by allowing or directing a mutation of the mating type alleles in the direction a → α or α → a, during or soon after the germination of a single haploid ascospore of either a or α mating type. Thus, from a single haploid ascospore, a mixed population of a and α haploid cells develop, which can conjugate to yield sporulation competent a/α diploids, isogenic for all loci except mating type. It is of interest that heterothallic strains can become homothallic through mutation (Hopper and Hall, 1975).

Roman and Sands (1953) showed that heterozygosity for the mating type allele, which is located on chromosome III, is a necessary prerequisite for the occurrence of meiosis and hence of sporulation. That is, the gene is not only concerned with mating, but also with the sporulation process. Using an a/α haploid disomic strain of S. cerevisiae, Roth and Fogel (1971) reported that cells require the simultaneous presence of both a and α alleles for premeiotic DNA synthesis. Haploid strains of either a or α mating type, and diploid

strains homozygous (a/a or α/α) for the mating type alleles cannot undergo premeiotic DNA synthesis, and consequently recombination, nuclear division by meiosis and ascospore formation do not occur (Roth and Lusnak, 1970). Diploid strains heterozygous (a/α) for the mating type alleles are competent to undergo premeiotic DNA synthesis, so that recombination, meiotic nuclear division and ascospore formation follow.

Nutritional Requirements of the Phases in the Life Cycle of *Saccharomyces*

The alternation of haploid and diploid phases in the life cycle of typical four-spored *Saccharomyces* is dependent upon the nutritional conditions to which the cells are exposed. De Seynes (1868), who was the first to observe endospore formation in yeast, noted that it occurred when the growth medium became depleted of nutrients. Subsequently, others found that nutritional deprivation caused *Saccharomyces* cells to cease growth and to differentiate into asci containing refractile spores (Engel, 1872; Stantial, 1935). Generally for maximal yields of asci, sporulation competent cells of *Saccharomyces* should be nourished at 27°C in a nutrient rich growth medium consisting of sources of nitrogen, carbon, vitamins and inorganic salts, and then allowed to respire in a nitrogen-free sporulation medium, containing a suitable carbon source such as acetate. After transfer to the sporulation medium, the cells cease growth, and undergo meiosis followed by ascospore formation (Miller and Hoffmann-Ostenhof, 1964). However, some increase in cell numbers does occur in the sporulation medium because developing buds on cells of sporulation competent *Saccharomyces* complete nuclear division by

mitosis, and separate from the parent cells before the latter sporulate (Esposito and Esposito, 1974). If the asci are returned to nutrient rich conditions, the ascus wall degenerates and each of the ascospores swells and germinates. During germination diploidization can occur through fusions between cells of opposite mating type. Normally spores of opposite mating type fuse directly within the ascus prior to budding.

Factors Influencing the Number of Spores Per Ascus

As indicated above, under certain conditions vegetative cells of sporulation competent *Saccharomyces* differentiate into tetranucleate asci, the nuclei constituting the haploid products of meiosis, but not each of the haploid nuclei necessarily becomes enclosed within a spore wall. A maximum of four spores per ascus can be expected, but asci containing one, two, or three spores are often found. The cytological observations of Nagel (1946), and Pontefract and Miller (1962) showed unenclosed nuclei in the epiplasm between the spores and ascus walls of asci containing fewer than four spores.

Nutritional conditions may have a marked influence on the number of spores per ascus. Lindegren and Lindegren (1944) found that a sporulation medium prepared from potato favoured two-spored ascus formation, whereas on beet medium most asci were four-spored. Miller (1957) found that the kind and concentration of carbon source in the sporulation medium had a marked effect on the number of spores per ascus. Thus, with acetate or pyruvate there were more spores per ascus than with glucose, fructose, mannose, galactose or dihydroxyacetone. There were more spores per ascus at the higher concentrations of these

substrates, especially with acetate and pyruvate. When yeast cells sporulate in distilled water or buffer in the absence of a carbon source, few asci develop and these are mostly two-spored (Miller and Halpern, 1956; Miller, 1957; Vezinhet, 1969). The addition of assimilable nitrogen compounds to acetate sporulation medium decreases total sporulation (Miller, 1963). Some nitrogen sources (casein hydrolysate, glutamic acid, ammonium sulfate) decreased the average number of spores per ascus, whereas others (valine, tyrosine, isoleucine, reduced glutathione) increased it. Kleyn (1954) added various nitrogen compounds to a dilute complex sporulation medium and noted that with aspartic acid 99% of the asci formed were two-spored. But when cells were sporulated in the same sporulation medium supplemented with glycine, he obtained 92% three and four-spored asci. Fowell (1967) showed that varying the cell density of S. cerevisiae in sporulation medium could influence the extent of four-spored ascus formation. A narrow optimum cell density range for maximum four-spored ascus formation was evident. The pH of the sporulation medium influenced four-spored ascus production: strains of S. cerevisiae displayed either a broad or a narrow pH optimum for maximum numbers of four-spored asci. Some of Fowell's strains produced no four-spored asci on sodium pyruvate agar sporulation medium, but the addition of a potassium salt made four-spored ascus development possible. With other strains, which could produce some four-spored asci on sodium pyruvate slants, the potassium salt addition gave a marked increase in four-spored ascus yields.

The composition of the sporulation medium may also induce some strains of _S. cerevisiae_ to form more than four spores per ascus. Santa María (1959) obtained seven isolates that produced up to eight spores per ascus in a sporulation medium devised by Kleyn (1954) containing glucose, sodium acetate, sodium chloride and tryptose, but in sporulation medium with acetate alone the maximum was four per ascus. Patel and Miller (1972) observed a small proportion of asci with more than four spores per ascus in acetate sporulation medium containing glycerol.

The composition of the medium in which the cells are grown may affect their ability to form three and four-spored asci after transfer to sporulation medium. Tremaine and Miller (1954) reported an increase in four-spored ascus development in an acetate sporulation medium, when an isolate of bakers' yeast was grown in a defined presporulation medium supplemented with the vitamin inositol. Fowell and Moorse (1960) showed that increasing the glucose concentration in Lodder-Rij's presporulation medium increased the proportion of four-spored asci formed after transfer to acetate agar, and that aeration of presporulation cultures gave a marked increase in yields of four-spored asci as compared with cells grown anaerobically. Halbach-Keup and Ehrenberg (1971) reported that _Saccharomyces_ cells transferred to sporulation medium during the transitory phase between the fermentative and respiratory stages of the growth curve gave the highest yields of four-spored asci.

It is possible to determine the number of buds a yeast cell has produced, and hence its age, by fluorescence staining of bud scars with

calcofluor. Sando and Saito (1970) found that older cells which had budded several times produced three and four-spored asci, whereas freshly formed cells which had not yet produced daughter cells invariably differentiated into two-spored asci.

It is well known that the extent of four-spored ascus production in any sporulation medium varies from strain to strain of *S. cerevisiae* (Kirsop, 1956). Furthermore, within a strain, single cell isolates may show differences in the yields of four-spored asci in sporulation medium (Fowell and Moorse, 1960).

As mentioned above, Nagel (1946) and Pontefract and Miller (1962) demonstrated that incomplete asci, i.e. those with less than four spores, have non-enclosed nuclei visible in the epiplasm. This cytological evidence suggests that since four nuclei were formed, the nuclei in all the ascospores were haploid. Magni (1958), Takahashi (1962) and Takahashi and Akamatsu (1963) have confirmed this genetically by isolation of single spores followed by mating, sporulation of the resulting diploids, and testing for segregation of markers. It does not follow, however, that reduction division occurs in the asci of all strains of *S. cerevisiae* that produce less than four spores per ascus.

Unusual Saccharomyces with Two-Spored Asci

Grewal and Miller (1972) examined cytologically 17 strains of *S. cerevisiae* which formed few or no asci with more than two spores. Fourteen of the strains underwent apparently normal meiosis during sporulation, since the asci always contained four nuclei. Three of the strains were unusual, in that the asci never had more than two nuclei.

From a detailed study, they reported that these strains (19e1, ATCC-4117, ATCC-4098) were unlike typical *S. cerevisiae* yeasts in the following respects:

(i) Asci containing only two spores were consistently produced in acetate sporulation medium.

(ii) Sporulating cells never contained more than two nuclei.

(iii) The nucleus within each ascospore was apparently diploid.

(iv) Ascospores were never observed to conjugate during germination, which the haploid spores in four-spored asci normally do.

(v) Clones derived from germinated single spores were competent to sporulate.

(vi) Unlike meiosis I, the single equational nuclear division that preceded ascospore formation was not glucose repressed (Miller, 1964).

(vii) The two spores in each ascus were joined by a conspicuous "intersporal body".

The life cycle of these unusual two-spored strains of *Saccharomyces* is shown in Figure 2.

Grewal and Miller at first assumed that the nuclear division in the asci was mitosis. But when an unpublished cytological examination by Dr. C.F. Robinow using the Giemsa stain indicated that the nuclear division had some of the morphological characteristics of meiosis I, they decided to refer to it as equational.

Seventy-five years ago Guilliermond (1903, 1905) made cytological observations on nuclear division during sporulation of a

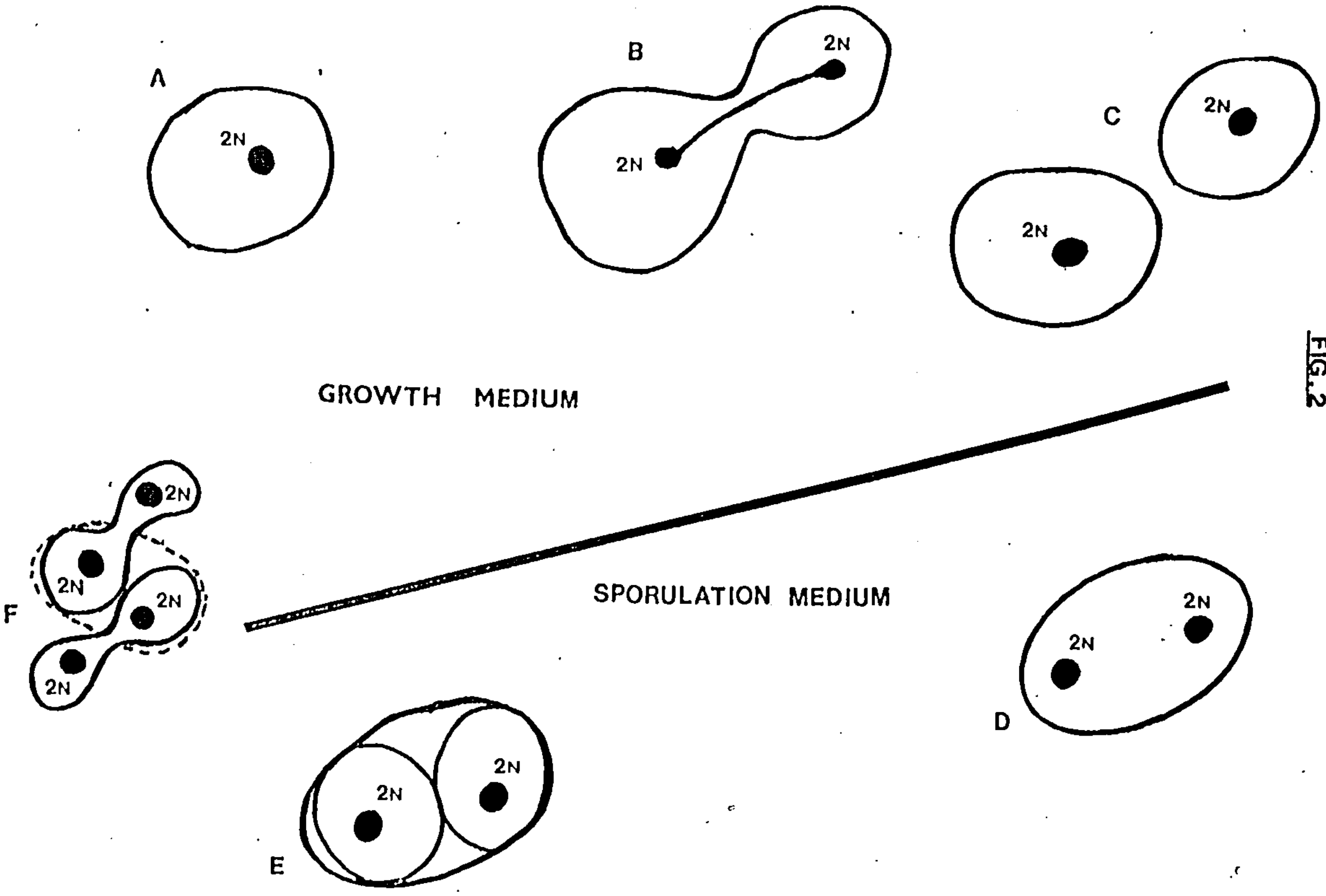
Figure 2: Life cycle of unusual two-spored strains of *Saccharomyces cerevisiae* which do not undergo reduction division during sporulation (after Grewal, 1972).

A, B, C: Vegetative nuclear division by budding during the growth phase

D, E : A single equational nuclear division yields two diploid nuclei preceding ascospore formation. Consequently, two-spored asci are produced in the sporulation medium

F : Germination of the ascospores without conjugation

FIG. 2



two-spored strain of *Saccharomyces pastorianus*. Sporulating cells never contained more than two nuclei, and thus a single nuclear division preceded ascospore formation. Furthermore, he observed no conjugations during ascospore germination.

In an ultrastructure study of strains 19e1 and 4117, Moens (1974) found that the single nuclear division which preceded ascospore formation resembled the nuclear behaviour of four-spored yeast strains during sporulation. As in typical four-spored *Saccharomyces*, a round, granular body was observed in association with the nucleoli of sporulating cells after 4 hours in the sporulation medium. In sporulating four-spored *Saccharomyces*, this structure is known to contain synaptinemal complex-like elements (Moens and Rapport, 1971), and the time at which this structure is evident corresponds to the onset of premeiotic DNA synthesis (Croes, 1966). After 8 hours had elapsed in the sporulation medium, which corresponds to the end of premeiotic DNA synthesis in typical four-spored *Saccharomyces*, he noted that nuclear division in both strains began with a spindle which could have been either a mitotic or a first meiotic spindle, but suddenly the spindle, the spindle pole bodies and nucleus took on features characteristic of meiosis II in four-spored *Saccharomyces*. In addition, he noted that at the completion of the single nuclear division and ascospore formation, the parental nucleolus was discarded in the epiplasm surrounding the two ascospores, which never occurred in association with nuclear division by mitosis. The abandonment of the nucleolus on completion of sporulation is characteristic of yeast strains that undergo two "normal" meiotic divisions (Moens, 1971).

Nevertheless, Moens did not conclude that the single nuclear division was meiotic.

In a more recent study, Moens *et al*. (1977) combined genetical and cytological approaches to study the manner of nuclear division during sporulation of the 4117 strain. In this regard, the nucleolus-associated granular body was observed in sporulating cells after 4 hours in the sporulation medium, but synaptinemal complexes were rare. Using a force-mating technique devised by Morrison *et al*. (1975), they mated 4117 with a haploid adenine-requiring strain and sporulated the resulting triploids from the mated cells. Ascospores dissected from the resulting asci yielded diploid colonies which produced two-spored asci like the original 4117 strain. However, these synthesised diploid clones showed abundant synaptinemal complexes and meiotic levels of recombination during sporulation. In their opinion, this was evidence that: (1) a normal meiotic prophase was retained in the original 4117 strain, (2) following synapsis and recombination, desynapsis occurred, (3) duplicated homologues failed to segregate at anaphase of meiosis I and (4) meiosis II occurred with division of the centromeres and chromosomes. They also proposed that strain 4117 was apomictic because its sexual cycle bypassed gamete formation as in apomictic plants.

Apomixis

Plants and animals were classified by Winkler in 1908 into three groups according to their mode of reproduction. Those organisms in which he believed sexual differentiation and fertilization had not yet arisen were called amictic (Gk. a-without, mixis-mingling). Sexually differentiated organisms were designated mictic. In the

mictic category, organisms occasionally arose in which reproduction was not associated with fertilization, and in which the latter process was in fact absent. These organisms were called apomictic (Gk. apo-from or away). Thus apomixis was a derived state in which the fertilizing mechanism had been lost.

In the plant kingdom seed formation without fertilization was first observed by Smith in 1841 in female plants of *Alchornea ilicifolia*. Braun (1857) confirmed Smith's observations, and also reported an alga (*Chara crinita*) in which the egg cell developed without prior fertilization. Subsequently, egg cell development in the absence of fertilization was found in many higher plants, and this subject has been extensively reviewed by Gustafsson (1946, 1947 a,b), who discussed instances of apomixis in all major plant groups. Gustafsson's treatise on apomixis is the basic botanical work on the subject.

Recently, Palmer (1971) described a form of apomictic parthenogenesis in an ameiotic mutant of *Zea mays*, in which presumptive meiotic cells underwent what he considered to be a mitotic division instead of the normal meiotic divisions. The single equational nuclear division occurring within the meiocytes of sterile anthers shared features common to the first meiotic nuclear division in cells from fertile anthers, but there was no evidence of chromosome pairing in the sterile anthers.

Mittwoch (1978) briefly reviewed parthenogenesis or virgin birth in the animal kingdom. Apparently, a few parthenogenetically-derived chickens and turkeys have been hatched and were able to survive

to adulthood. The New Mexico whiptail lizard, _Cnemidophorus neomexicanus_ is a species hybrid consisting exclusively of females which reproduce parthenogenetically. The presumptive sexual cells undergo two rounds of DNA replication instead of one, and normal reductional nuclear division occurs, but the egg cell is diploid with the same chromosome complement as the mother. No cases of virgin birth have been authenticated in mammals. This subject is of interest in medicine. According to Mittwoch, there is strong evidence that ovarian tumours known as dermoid cysts originate from diploid cells which undergo meiosis I but not meiosis II.

Apomictic parthenogenesis in insects has been reviewed by Suomalainen _et al_. (1976). Apparently, chromosome pairing is lacking in some of these insects although transient pairing has been observed in others. Only one "maturation" division occurs in the eggs of apomictic parthenogenetic insects and this division is equational.

In the fungi, Gäumann (1928) used the term 'apomixis' to describe the development of sexual cells in the absence of copulation. He referred to the apomictic development of haploid cells as parthenogenesis, and the apomictic development of diploid cells as apogamy. Burnett (1956) modified Gäumann's definitions using the general term amixis to describe parthenogenesis in haploid organisms, and apomixis to describe parthenogenesis in diploid organisms where the events of sexual reproduction are lacking. In the present study, Burnett's definition of apomixis will be adopted.

Meiosis in the fungi always occurs in a differentiated cell e.g. ascus in Ascomycetes, basidium in Basidiomycetes, zygospore in

Phycomycetes. Yet certain members belonging to these taxonomic groups produce fruiting structures but bypass gamete formation within them. Instead, the nucleus divides equationally so that each of the resultant nuclei is identical to the original parental nucleus. Such is the case in multispored strains of the yeast *Lipomyces lipofer* (Henninger *et al*., 1974; Henninger and Emeis, 1974). In apomictic strains of this species, the nucleus does not undergo reduction division during sporulation, and instead, a series of equational nuclear divisions precedes ascospore formation within the ascus. In *Saccobolus versicolor* and *Peziza quelepidotia* (O'Donnell *et al*., 1976a,b), sexuality is apparently absent since they never observed antheridia but only ascogonia. *Pyronema domesticum* undergoes the apomictic production of ascogenous hyphae with formation of sterile apothecia following treatment with the herbicide, 2,4-D (Moore-Landecker, 1972).

Thus, the production of diploid progeny in sexual structures without fertilization is a well known phenomenon in plants, animals and fungi.

Objectives

From the foregoing it is evident that the 19e1 strain of *S. cerevisiae* is apomictic since it remains diploid throughout its life cycle, yet produces the normal sexual structure, the ascus. A single equational nuclear divison occurs in the ascus instead of normal reductional nuclear division. Thus, asci containing two uninucleate diploid spores are produced. However, Ashraf and Miller (1977, 1978a) were able to induce multispored asci containing more than two and sometimes as many as eight spores when the 19e1 strain was grown in

undefined presporulation media containing 0.1M of the herbicide amitrole. Clones derived from single spores isolated from the multispored asci were competent to sporulate, and they suggested that multiple equational nuclear divisions preceded ascospore formation in these asci. That is, under some circumstances, the 19e1 strain can produce asci containing more than the usual two spores, but not necessarily as a result of meiosis.

Except in the amitrole experiment, sporulation procedures with 19e1 using liquid culture techniques have always yielded exclusively two-spored asci (Grewal and Miller, 1972; Ho and Miller, 1978). However, Mr. K.H. Ho (personal communication, 1977) sometimes observed a few three and four-spored asci in agar slant sporulation cultures. In 1977, the writer sporulated 19e1 in 2% potassium acetate solution following growth in a defined liquid medium (Yeast Nitrogen Base) supplemented with 2% instead of the usual 1% glucose, and a few three and four-spored asci (~ 5%) were obtained. The previous workers (Grewal and Miller, 1972; Ho and Miller, 1978) using 1% glucose, had not observed such asci and the writer confirmed this.

It is apparent that alterations in nutritional or other cultural conditions can enable the 19e1 strain to produce asci with three or four spores. A study was therefore undertaken to investigate the effect of various environmental conditions in both presporulation and sporulation cultures on three and four-spored ascus formation. A further objective was to determine whether the nuclear divisions in such asci were meiotic or equational.

MATERIALS AND METHODS

Stock Cultures

One of the two-spored strains of *Saccharomyces cerevisiae* (19e1) described by Grewal and Miller (1972) was employed in this study. Stock cultures were maintained on slants of Wickerham's (1951) Yeast Nitrogen Base (YNB) containing 2% glucose. The slants were prepared in the following way: Three grams Difco Bacto agar were dissolved in 100-ml glass-distilled water and 9-ml volumes of the agar solution were transferred to 15mm test tubes. One ml of a 10X solution of Difco Yeast Nitrogen Base containing 20% glucose was added to each of the test tubes and mixed thoroughly. The test tubes were autoclaved for 15 min at 121°C and 15 psi pressure. After inoculation, the slants were incubated for 2 days at 27°C and were then stored at 4°C. Fresh stock cultures were made every 3 to 4 weeks.

Presporulation Phase

Two types of medium were used to grow cells for inoculation of sporulation cultures:

(i) Yeast Nitrogen Base Medium (YNB):

The composition of this defined presporulation medium is given in Appendix I. This medium, which lacks a carbon source, contains vitamins, trace elements, three amino acids in trace amounts, ammonium sulfate and other salts in known quantities. The medium was prepared either directly in final strength by dissolving 0.67 gm Difco Yeast Nitrogen Base in 100ml deionized glass-distilled water, supplemented with the desired final concentration of glucose as the carbon source,

or as a 10X strength stock solution supplemented with 10X the required glucose concentration. The YNBG solutions were sterilized by filtration through Amicon membrane filters (47mm, pore diameter 0.45 µm), and stored at 4°C in autoclaved 1,000-ml erlenmeyer flasks. For experimental use, 50-ml volumes of YNBG medium were dispensed aseptically into autoclaved 250-ml erlenmeyer flasks. In cases where the 10X strength stock medium was used, a 1:9 dilution with sterilized deionized glass-distilled water was necesary to obtain the final medium. Modifications to the YNB medium were made according to experimental requirements.

(ii) Malt extract-yeast extract-peptone-glucose medium (MYPG): This is an undefined medium having the following ingredients per liter of distilled water:

3 gm	Bacto malt extract
3 gm	Bacto yeast extract
5 gm	Bacto peptone
1 gm	KH_2PO_4
20 gm	glucose

The MYPG growth medium was devised by Wickerham (1951) and modified by Patel and Miller (1972) who increased the glucose concentration to 2% and added KH_2PO_4 to lower the pH to 5.4±0.2. Fifty-ml volumes of this medium were poured into 250-ml erlenmeyer flasks. The flasks were sealed with aluminum foil and autoclaved 15 min. After cooling to room temperature, each flask was plugged aseptically with sterile sponge foam plugs (Dispo) and stored in the dark.

Prior to each experiment, a cell inoculum was transferred from

the YNBG stock culture to a fresh YNBG agar slant, and incubated 24 hr at 27°C. The 50-ml volumes of growth medium in sterile 250-ml flasks were inoculated with cells from the 24 hr slant culture at an initial cell density of 10^5/ml of the medium. Cell counts were made with Spencer "Bright-Line" haemocytometer counting chambers. The growth flasks were incubated at 27°C for 21 hr in a Warner-Chilcott Laboratories model 2156 water-bath shaker operated at 100 oscillations per minute.

Sporulation Phase

After 21 hours incubation, the vegetative cells were harvested from the growth medium by centrifugation in a Beckman Model J-21 centrifuge at 3,020g and 5°C for 15 minutes. The cells were washed twice with sterilized deionized glass-distilled water and resuspended in it. An appropriate volume of cell suspension was then transferred to 50-ml volumes of autoclaved potassium acetate solution of known concentration, to give a cell density of 10^7/ml. Sporulation cultures were incubated in the same manner as the presporulation flasks but for a longer time: 48 hours. The percentage of cells that sporulated and the percentage of cells containing 2, 3, and 4 ascospores were determined by scoring 500 entities from each culture flask. Each experiment was done in quintuplicate and all experiments were repeated unless otherwise indicated.

Giemsa Stain Technique

The Giemsa procedure employed was that described by Ramirez and Miller (1962). A drop of yeast suspension was placed on a cover glass (22mm round, No. 1) bearing a thin layer of Mayer's albumen (Gurr,

1962), and allowed to dry for a few minutes. The cover glass bearing the preparation was placed in freshly prepared Helly's fixative (Gurr, 1962) for 20 min, and was then rinsed with 70% ethanol, and placed in a Columbia staining jar containing 1N HCL for 10 min at 60°C. The hydrolysis was immediately halted by dipping the cover glass in cold water. The rinsed cover glass was submerged for 15 min in a freshly prepared staining solution consisting of 1 drop Gurr's R66 Giemsa stain per ml of Gurr's 0.5M, pH 6.8 phosphate buffer. The stained preparation was rinsed rapidly with cold tap water, and pressed onto a slide to flatten the cells. The edge between the coverslip and the slide was sealed with Permount (Fisher Scientific Co.) to prevent drying.

Spore Germination

The germination medium used was devised by Brock (1961), and has the following composition per liter glass-distilled water:

5gm glucose

1gm $MgSO_4.7H_2O$

1.4gm KH_2PO_4

This medium was modifed by the addition of 30gm Difco Noble agar, sterilized by autoclaving, and then poured aseptically into autoclaved 15X100mm glass petri dishes to a depth of approximately 2mm per dish. Once the medium solidified, moisture condensation was evaporated from the plates by stacking them in a 27°C incubator overnight. They were then stored in sealed plastic bags at 4°C until needed.

The culture technique used by Miller (1970) to observe the germination of yeast ascospores was employed except that Brock's mating

medium with 3% Difco Noble agar was used for plate preparation instead of MYPG with 5% Fisher Agar SP. Asci from 48 hr sporulation cultures were harvested by centrifugation and washed twice with sterilized glass-distilled water. The population density of the ascus suspension was determined with haemocytometer counting chambers, and an appropriate dilution was carried out to give a final density of $10\text{-}20 \times 10^6$ asci per ml in sterile glass-distilled water. A loopful of suspension was then placed on the agar surface and immediately covered with a sterilized cover glass (22mm round, No. 1), which was pressed down to spread the suspension evenly over the area beneath it. Six samples were seeded on each petri dish in this way. The plates were incubated at $27^{\circ}C$ and observations were made with the light microscope between 6 and 12 hours. Miller (1970) found that this procedure facilitated the observation of conjugation, since the germination of all spores in an ascus takes place in one plane between the cover glass and agar.

Micromanipulation of Asci

For genetic analysis of yeast ascospores, the micromanipulative technique designed by Ashraf and Miller (1978b) was used with some modifications. A dilute Glusulase-treated ascus suspension was seeded on the surface of a hanging drop of water agar (2% Difco Noble agar) instead of MYPG agar, to avoid premature germination of ascospores. Single asci were then transferred to the surface of hanging drops of the following medium:

0.67gm	Difco Yeast Nitrogen Base
2gm	glucose

0.1gm	Difco yeast extract
2gm	Difco Noble agar
100ml	deionized glass-distilled water

The asci were dissected on the surface of these drops, and the separated spores were allowed to grow for 2-4 days before transfer to MYPG agar slants. Another modification was the substitution of copper rings (15mm wide, 10mm long, 1mm thick) for the non-autoclavable acrylic plastic rings used to support the coverslips bearing hanging drops in the petri dishes. In this way, the rings could be autoclaved with the moistened filter paper in the petri dish prior to the preparation of hanging drops, without the need to sterilize them separately with 70% ethanol.

Chemicals

The following analytical grade reagents were obtained from the J.T. Baker Chemical Company:

ammonium sulfate
glucose (anhydrous dextrose)
potassium acetate
zinc acetate
zinc sulfate

The following analytical grade chemicals were obtained from the Fisher Scientific Company:

acid boric
magnesium sulfate
manganese (ous) sulfate
L-tyrosine
potassium iodide
potassium phosphate (monobasic)
sodium chloride
sodium molybdate

The following vitamins were used in this study:

+-biotin

+-calcium pantothenate
folic acid
inositol
para-aminobenzoic acid
pyridoxine hydrochloride
riboflavin
thiamine hydrochloride

All the vitamins were supplied by the Calbiochem-Behring Corporation except inositol and para-aminobenzoic acid which were obtained from Difco Laboratories.

"Vitamin-free" casein hydrolysate was supplied by Nutritional Biochemicals Corporation (Cleveland, Ohio).

Glusulase was supplied by Endo Laboratories Inc. (Garden City, N.Y.).

Error bars in all figures indicate standard deviation about the mean.

RESULTS

Effect of Varying the Concentration of Carbon Source in Growth and Sporulation Medium on Sporulation

As mentioned in the Introduction, a few 3 and 4-spored asci were produced by the 19e1 strain, when the glucose concentration in presporulation medium (PSM) was changed from 1 to 2%. An experimental series was therefore undertaken in which the glucose concentration in the PSM was varied from 1 to 8%. Also, the concentration of potassium acetate in the sporulation medium (SM) was varied, from 0 to 5%.

The results are shown in Figure 3. The highest yields of 3 and 4-spored asci were obtained with 6% glucose in PSM and 3 to 3.5% acetate in SM. With 2% glucose in PSM, the highest yield resulted at 4.5% acetate in SM. With 8% glucose (not shown in graph), the results were similar to 6%. The increased yield obtained by increasing the glucose content of the PSM from 1 to 6% is quite striking. It is interesting that with 6% glucose in the PSM, the highest acetate concentration suppressed 3 and 4-spored ascus production, but not with 1% glucose.

The experiment was repeated in part substituting the undefined medium MYPG for YNBG as PSM. One, two, and six percent glucose were included in MYPG and the cells were transferred to 2, 3, 3.5 and 5% potassium acetate for sporulation. Asci with more than 2 spores were never observed. Obviously, factors other than carbon sources must be involved in determining 3 and 4-spored ascus production.

Effect of Changing the Cell Density In Potassium Acetate SM

Figure 3: Effect of varying the concentration of carbon sources in growth and sporulation media on 3 and 4-spored ascus production.

Fifty-ml volumes of YNB presporulation media consisting of 0.67% Difco Yeast Nitrogen Base supplemented with increasing glucose concentrations were inoculated with cells from 24 hr slant cultures at an initial cell density of 10^5/ml, and aerated at 27°C for 21 hr in a water-bath shaker operated at 100 oscillations per min. The vegetative cells were harvested from the YNBG presporulation medium and washed twice with sterile glass-distilled water and resuspended at a cell density of 10^7/ml in 50-ml volumes of potassium acetate sporulation medium of known concentration (horizontal axis). Sporulation cultures were maintained in the same way as the growth flasks except for a duration of 48 hr. The mean percentages of 3 and 4-spored asci were determined by scoring 500 entitites from each of 5 replicate flasks. The experiment was done twice.

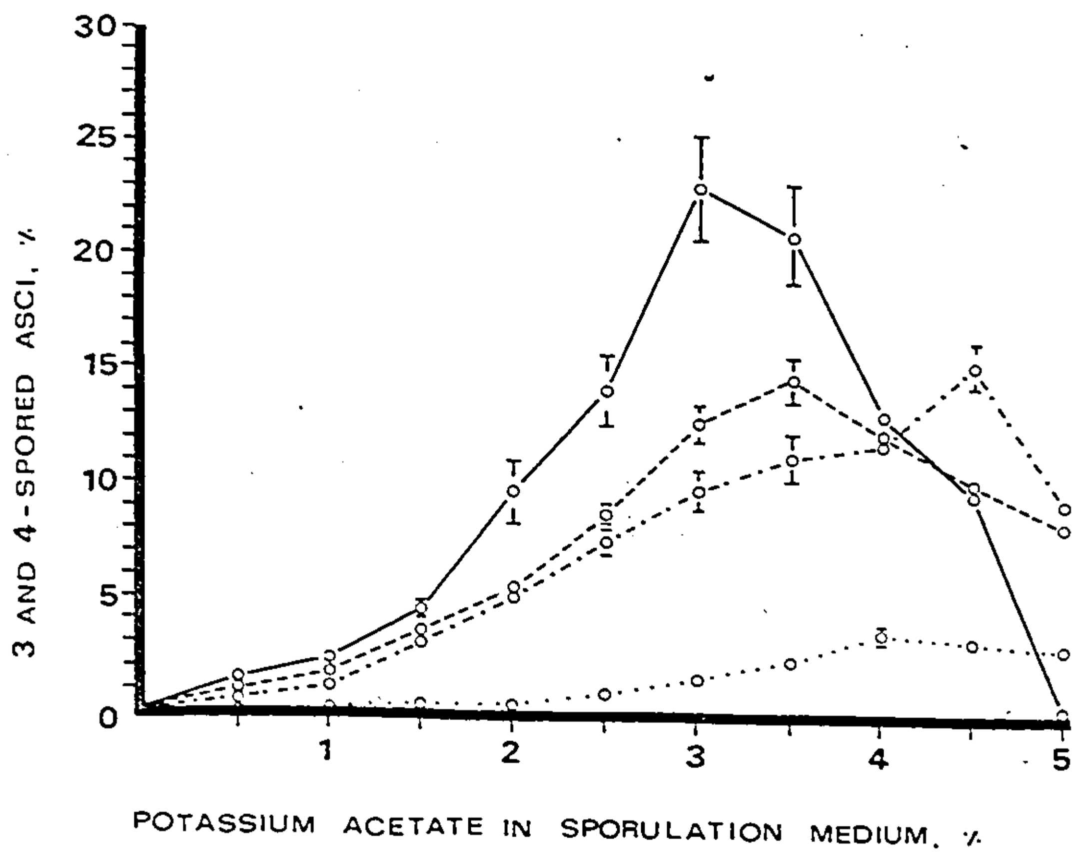
· · · · · · 1% glucose

-·-·-·- 2% glucose

------- 4% glucose

———— 6% glucose

FIGURE 3



Fowell (1967) showed that some yeast strains gave greater yields of 4-spored asci when the cell population density in the SM was low, whereas others gave greater yields when the cell population density in SM was higher. To determine whether a trend would be observed with the 19e1 strain in regard to 3 and 4-spored ascus production, cells were grown in YNB PSM supplemented with 6% glucose, and sporulated at different cell densities in 2% potassium acetate SM. These results are shown in Figure 4. More 3 and 4-spored asci were formed when the cell density in SM was 1 million/ml than at the higher cell densities.

Effect of An Inhibitor of Meiosis on Sporulation

Miller (1964) showed that the addition of glucose to SM inhibits meiosis in "normal" four-spored *Saccharomyces* but Grewal and Miller (1972) found that glucose treatment gave little inhibition of sporulatioh with 19e1.

The effect of glucose on sporulation was determined under conditions that gave high yields of 3 and 4-spored asci (6% glucose in YNBG PSM and 2% potassium acetate in SM). To the SM were added, 0, 0.125, 0.25, 0.5, and 1% glucose. The effect on total yields of asci and on 3 and 4-spored ascus production is shown in Figure 5. Even the lowest concentration of glucose used strongly suppressed 3 and 4-spored ascus production but had slight effect on total sporulation.

Giemsa Staining of Sporulating Cells

Since asci in the 19e1 strain normally contain only 2 nuclei (Grewal and Miller, 1972), it was of interest to determine whether treatments that induced 3 and 4-spored ascus formation would also yield

Figure 4: Effect of varying the cell density in 2% potassium acetate sporulation medium on the production of 3 and 4-spored asci.

Fifty-ml volumes of growth medium consisting of 0.67% YNB supplemented with 6% glucose and inoculated with 10^5 cells/ml were incubated 21 hr at 27°C in a water-bath shaker operated at 100 oscillations per min. The vegetative cells were harvested from the growth medium and appropriate volumes of cell suspension were transferred to 50-ml 2% potassium acetate sporulation medium to give cell densities of 1, 5, 10, 15 and 20 million/ml. The sporulation cultures were incubated in the same way as the growth flasks except for a duration of 48 hr. The mean percentages of 3 and 4-spored asci were determined by scoring 500 entities from each of 5 replicate flasks. The experiment was done twice.

FIGURE 4

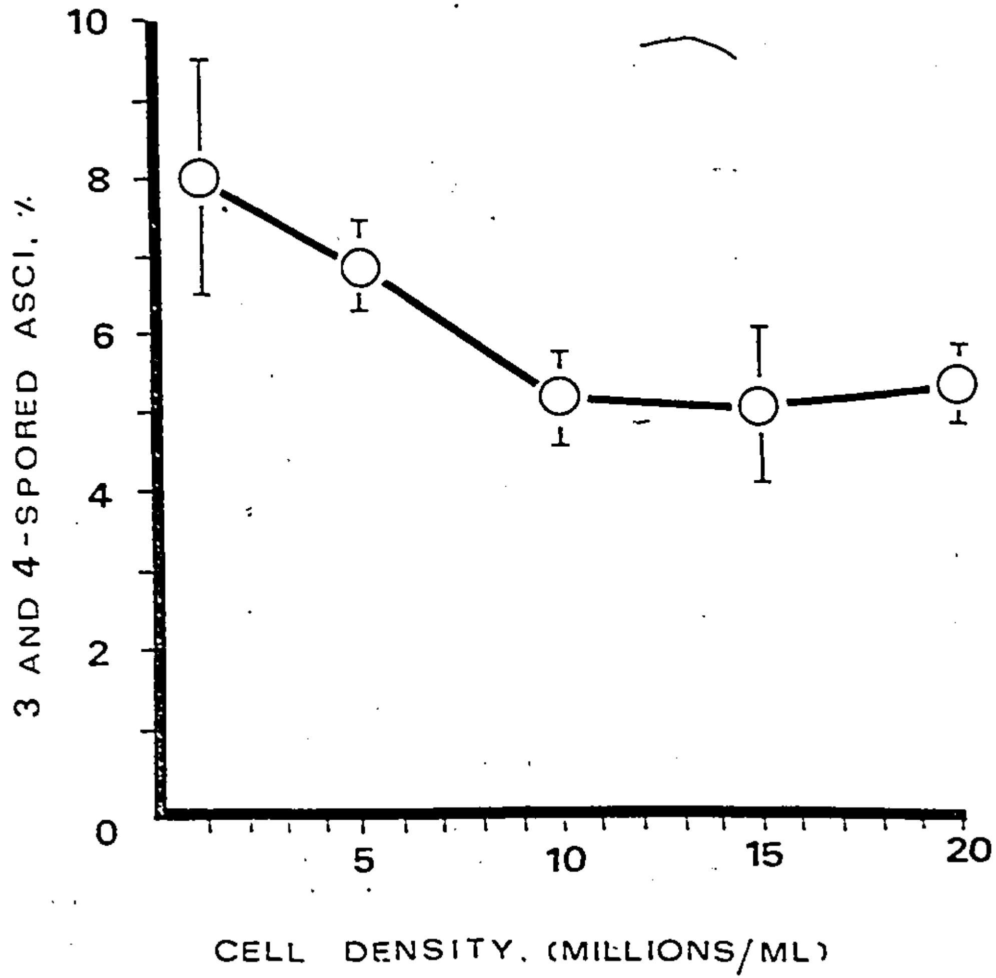
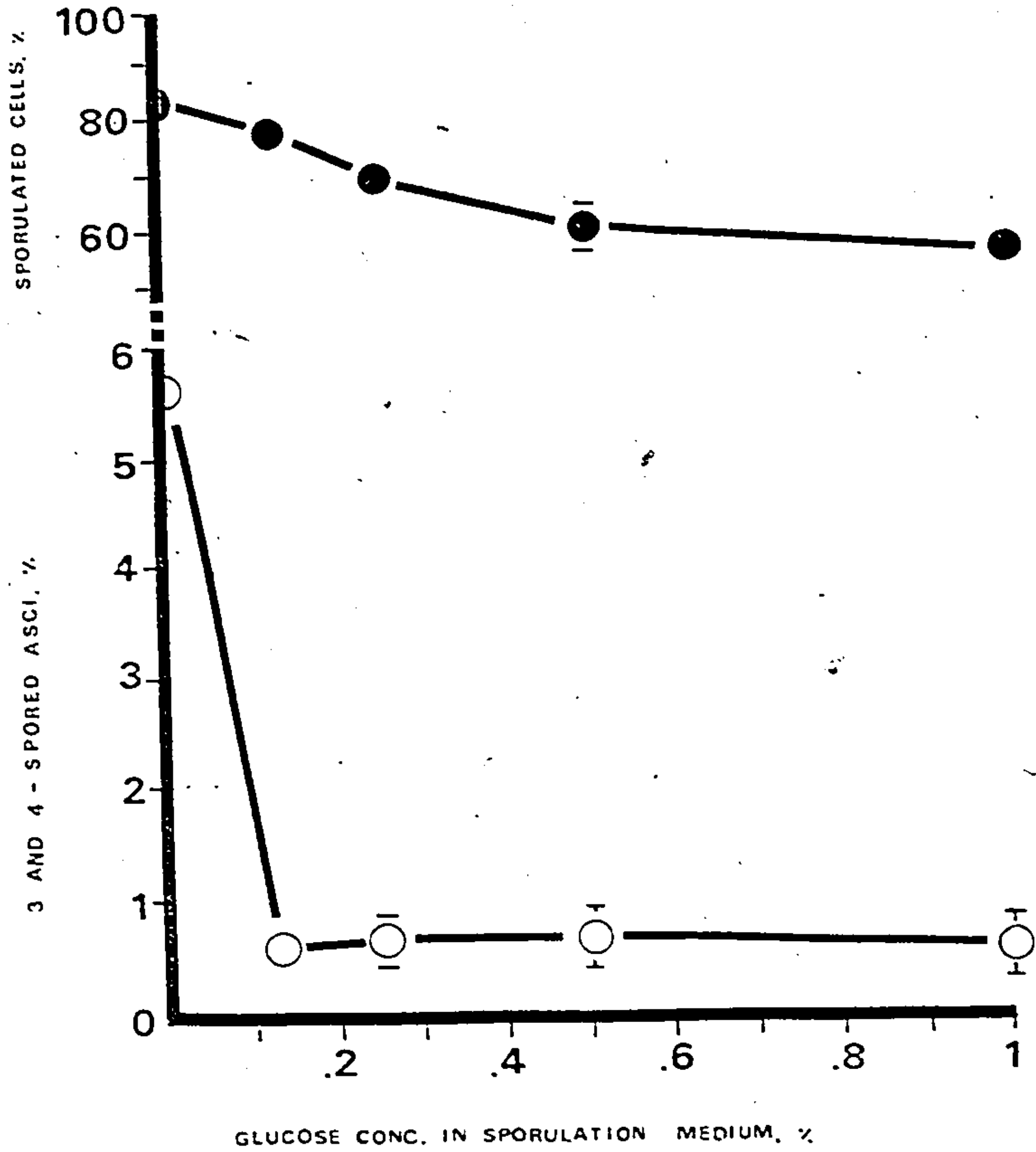


Figure 5: Effect of addition of glucose to potassium acetate sporulation medium on total sporulation and on yield of 3 and 4-spored asci.

Cells were inoculated into 50-ml volumes of YNB supplemented with 6% glucose at an initial cell density of 10^5/ml and the growth culture flasks were incubated for 21 hr at 27°C in a water-bath shaker operated at 100 oscillations per min. Cells were harvested from the growth medium by centrifugation and washed twice in sterile glass-distilled water. Appropriate volumes of cell suspensions were transferred to 50-ml 2% potassium acetate sporulation media containing 0, 0.125, 0.25, 0.5 and 1% glucose to give a cell density of 10^7/ml. Sporulation cultures were maintained in the same way as the growth flasks, except that they were incubated 48 hr. The mean percentages of 3 and 4-spored asci were determined by scoring 500 entities from each of 5 replicate flasks. The experiment was done twice.

FIGURE 5



2-spored asci which contain more than 2 nuclei. Cells grown in the YNB growth medium supplemented with 6% glucose were transferred to 3.5% potassium acetate SM and after 18 hr, cell samples were washed in sterile deionized glass-distilled water and then Giemsa stained. The reason that cells were taken from 18 instead of 48 hr sporulation cultures is that unenclosed nuclei tend to degenerate with time. The experiment was done twice and 5 slides were made each time.

Two-spored asci with unenclosed nuclei were not observed. One-spored asci always contained 2 nuclei, one of which was unenclosed (Figure 6A). Three-spored asci contained 3 nuclei in spores, and 1 unenclosed nucleus (Figure 6B). Thus two classes of asci existed in respect to the number of nuclei per ascus: binucleate and tetranucleate asci. Asci containing more than four nuclei were not found. Hence, 1 and 2-spored asci were in the binucleate class, and 3 and 4-spored asci in the tetranucleate class.

Observations on Germinating Ascospores

To observe the germination of ascospores microscopically, drops of a dilute suspension of asci obtained from 48 hr 3.5% potassium acetate sporulation cultures were transferred to the surface of Brock's (1961) mating medium modified by the addition of 3% agar, and the seeded areas covered with sterile coverslips.

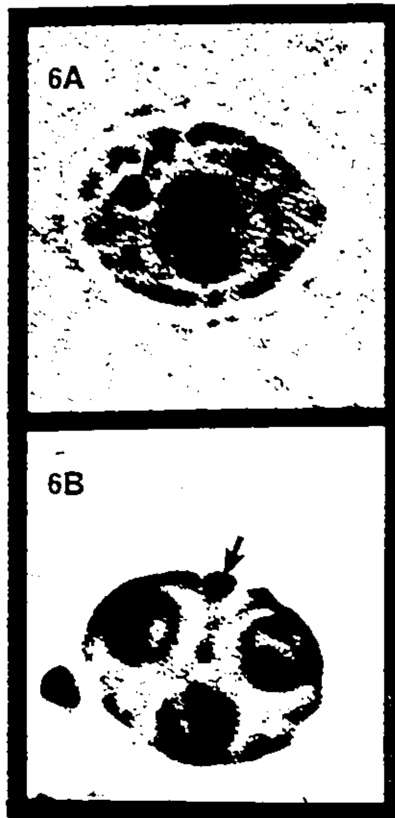
Conjugations between cells from germinated ascospores were never observed in strain 19e1 by Grewal and Miller (1972), and in the present study, this was true of 2-spored asci. However, cells from germinated ascospores derived from 3 and 4-spored asci were observed to conjugate, although infrequently. Conjugations occurred within the

Figure 6: Giemsa-stained sporulating cells.

Fifty-ml volumes of growth medium consisting of 0.67% YNB supplemented with 6% glucose were inoculated with cells at an initial cell density of 10^5/ml. At the end of a 21 hr incubation period at $27^\circ C$ in a water-bath shaker operated at 100 oscillations per min, the cells were harvested from the growth medium by centrifugation and washed twice in sterile glass-distilled water. An appropriate volume of cell suspension was transferred to 3.5% potassium acetate sporulation medium to give a final cell density of 10^7/ml. The sporulation cultures were maintained in the same way as the growth cultures, but after 18 hr incubation cell samples were removed and prepared for Giemsa staining by centrifugation and washing twice.

6A: Giemsa-stained binucleate ascus showing one nucleus within one ascospore and one unenclosed nucleus (arrow) in the epiplasm. 4,030x

6B: Giemsa-stained tetranucleate ascus showing one nucleus in each of three ascospores and one unenclosed nucleus (arrow) in the epiplasm. 3,185x


6A
6B

ascus during germination, or following germination (Figures 7A, 7B, 7C).

Genetic Analysis of Asci

Though 2-spored ascus production in 19e1 is not preceded by the normal two meiotic divisions, the glucose inhibition of 3 and 4-spored ascus production and the observation of conjugations when spores in such asci germinate indicate that normal meiosis occurs in these asci. If so, one would expect that single-spore cultures derived from these asci would not be competent to sporulate.

Fifteen asci were dissected (five 2-spored, five 3-spored and five 4-spored) and all the single-spore cultures when grown on MYPG slants sporulated with production of 2-spored asci. The cultures were inoculated on plates of MYPG agar and Difco Yeast Morphology agar (a medium of the same composition as YNBG except that it contains asparagine). Portions of the inoculated areas on the agar surface were covered with sterile coverslips and the dishes were incubated for 1 week. This is a standard technique in yeast taxonomy for observation of yeast growth characteristics.

All four single-spore cultures obtained from each 4-spored ascus were tested in this way. There was a 2:2 segregation of pseudomycelial: normal budding growth. Ratios of 2:1 and 1:2 for the same growth characteristics were obtained when the 3 single-spore cultures from each 3-spored ascus were tested. None of the single-spore cultures originating from 2-spored asci showed pseudomycelial growth. The same results were obtained on both media.

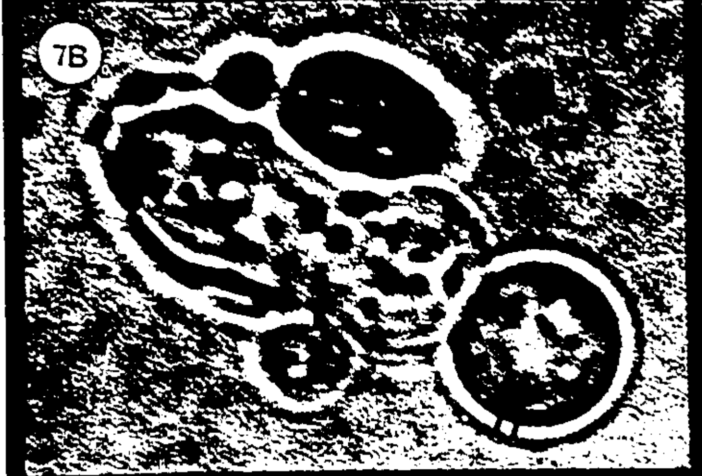
Figure 7: Germinating ascospores

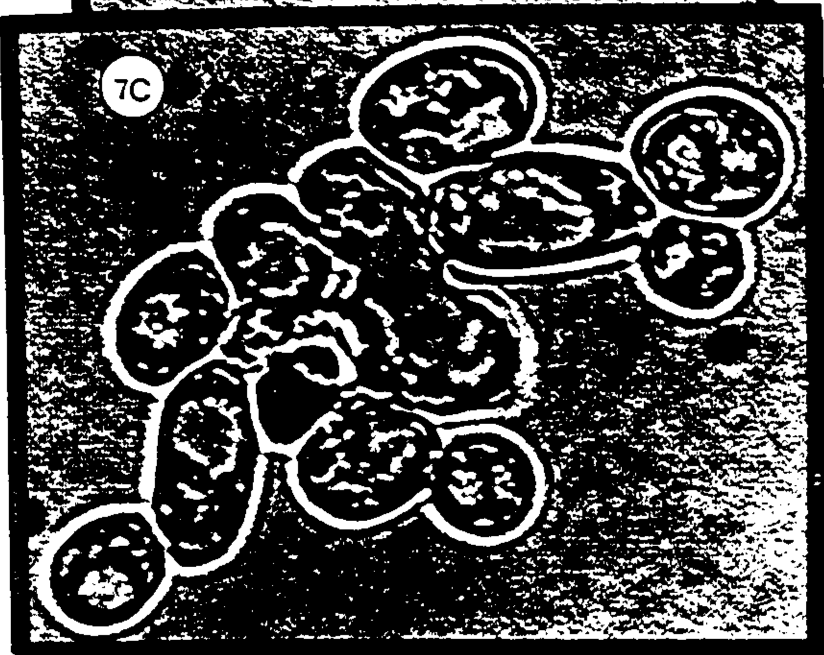
7A: Three-spored ascus after 6 hr in germination medium. Two of the spores are swollen and one ascospore (arrow) is not germinating. 13,750x

7B: Conjugation between two germinating ascospores (single arrow) ater 8 hr in germination medium. The zygote cell is producing a daughter cell (double arrow). 13,750x

7C: Conjugation between sister cells from a culture derived from a single ascospore after 10-12 hr in germination medium. 11,250x

7A


7B


7C

This is evidence that segregation has occurred in the 3 and 4-spored asci. The ability of the single-spore colonies derived from these asci to sporulate indicates that self-diploidization occurred.

Effect of adding MYPG Components and "Vitamin-Free" Casein Hydrolysate to YNBG Presporulation Medium

When the 19e1 strain was cultivated in the undefined PSM MYPG consisting of 0.3% Difco malt extract, 0.3% Difco yeast extract, 0.5% Difco peptone, 0.1% KH_2PO_4 and 6% glucose per 100 ml distilled water, the cells were unable to differentiate into asci containing 3 or 4 ascospores, and differentiated into predominantly 2-spored asci after incubation in 3.5% potassium acetate SM. Since asci containing 3 or 4 ascospores in addition to 2-spored asci were obtained in the same SM when cells were cultivated in the same way in the defined PSM YNB supplemented with 6% glucose, it was of interest to investigate which components of the MYPG medium inhibited the production of 3 and 4-spored asci.

The cells of strain 19e1 were cultivated for 21 hr in filter-sterilized media consisting of the YNBG medium with components of the MYPG medium added singly at the concentrations indicated above. Since Difco yeast extract and Difco peptone contain large quantitites of free amino acids, it was also of interest to cultivate the 19e1 strain in the YNBG medium supplemented with 0.1% "vitamin-free" casein hydrolysate.

The results are shown in Table 1. When yeast extract, peptone and "vitamin-free" casein hydrolysate were added to the chemically-defined YNBG growth medium, there was a reduction in 3 and 4-spored

Table 1

Effect of Addition of MYPG Components and "Vitamin-Free" Casein Hydrolysate to YNBG Growth Medium on Sporulation and 3 and 4-Spored Ascus Formation

Presporulation Medium	Total Sporulation,%*	3 and 4-Spored Asci,%*
0.67% YNB, 6% glucose (control)	86.8	4.9
0.67% YNB, 6% glucose, 0.1% KH_2PO_4	87.2	4.0
0.67% YNB, 6% glucose, 0.3% malt extract	87.6	4.4
0.67% YNB, 6% glucose, 0.5% peptone	94.4	0.6
0.67% YNB, 6% glucose, 0.3% yeast extract	86.5	0.1
0.67% YNB, 6% glucose, 0.1% casein hydrolysate ("vitamin-free")	86.0	0.3

* Cells were grown 21 hr and after 48 hr in 2% potassium acetate sporulation medium, percentages of total sporulation and of 3 and 4-spored asci were determined by scoring 500 entities from each of five replicate flasks. The experiment was done once.

ascus formation in SM. The addition of malt extract and KH_2PO_4 had no effect.

Yeast extract and peptone contain amino acids and other nitrogenous compounds in unknown quantities (Difco manual, 1967). Typical analyses of these components indicate a high percentage of L-tyrosine (personal communication, Difco Laboratories). The addition of 0.025% L-tyrosine to YNBG caused a 77% reduction in the percentage of 3 and 4-spored asci yielded in 2% potassium acetate SM, relative to the untreated control. Other components of the complex medium may also have an effect, but this approach was not pursued further.

Effect of Components of the Defined Medium Yeast Nitrogen Base (YNB) on 3 and 4-Spored Ascus Production

From the foregoing, it is obvious that MYPG grown cells did not yield 3 and 4-spored asci in potassium acetate SM. However, with cells grown in YNBG PSM, this was not the case. Since varying the concentration of glucose added to YNB had such a pronounced stimulating effect on 3 and 4-spored ascus production in SM (Figure 3), it was of interest to investigate the effect of varying the YNB content (i.e. all other constituents except glucose) in the PSM. The composition of Difco Yeast Nitrogen Base (YNB) is given in Appendix I.

The percentage of sporulation and the percentage of 3 and 4-spored asci formed in potassium acetate SM increased to a distinct optimum with increasing concentration of YNB in growth medium (Figure 8). Since total sporulation was high at the highest concentration of YNB, physiological conditions for sporulation were evidently maintained. The percentage of 3 and 4-spored asci decreased at the

Figure 8: Effect of varying the YNB content of growth medium containing 6% glucose on the % total sporulation and % 3 and 4-spored asci in varying concentrations of potassium acetate.

Cells were inoculated into 50-ml volumes of solutions containing 0.1, 0.5, 1, 1.5 and 2% YNB in 6% glucose at an initial cell density of 10^5/ml. The growth culture flasks were incubated for 21 hr at 27°C in a water-bath shaker operated at 100 oscillations per min. Cells from each growth medium were harvested by centrifugation, washed twice and an appropriate volume of cell suspension in each case was transferred to 50-ml potassium acetate sporulation medium to give a cell density of 10^7/ml. Sporulation cultures were maintained in the same manner as growth cultures but the incubation time was 48 hr. Total sporulation (open circles) and yield of 3 and 4-spored asci (closed circles) were determined by scoring 500 entities from each of 5 replicate flasks. The experiment was done twice.

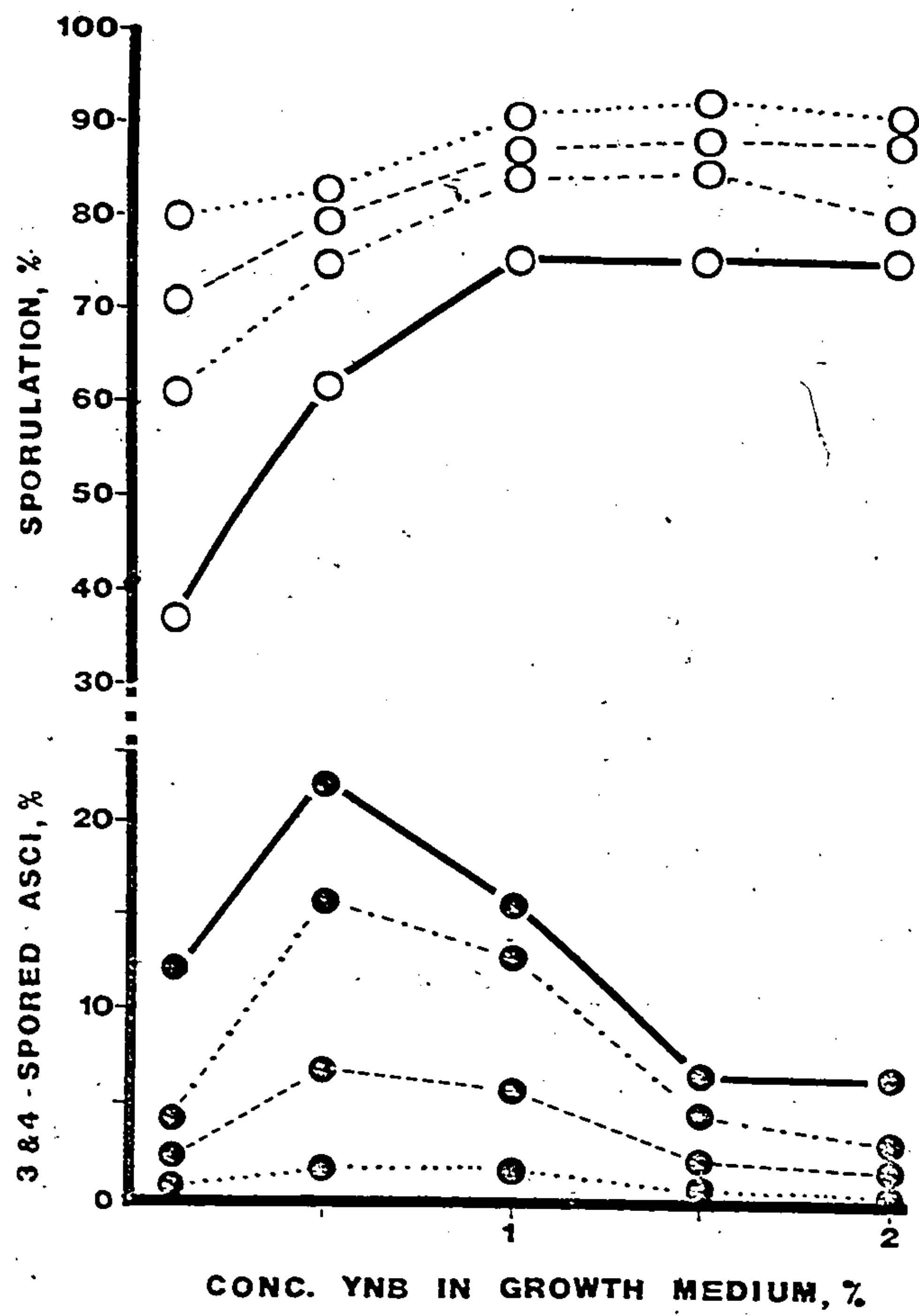
· · · · · · 1% potassium acetate

------ 2% potassium acetate

·-·-·- 3% potassium acetate

——— 3.5% potassium acetate

FIGURE 8



highest concentrations. The increase and decrease in the percentage of 3 and 4-spored asci may reflect the presence of stimulating and inhibitory factors in the YNB.

In a preliminary attempt to determine the stimulating and inhibitory components in YNB, solutions of the vitamins, salts, trace elements (see Appendix I), and ammonium sulfate were approximately tripled from their control values in 0.67% YNB. As shown in Table 2, this revealed both stimulating and inhibitory effects. When solutions of the trace elements and vitamins were added in this way, sporulation in 3.5% potassium acetate was depressed and the percentage of 3 and 4-spored asci increased. In comparison, the addition of ammonium sulfate decreased both percentages. When the salts concentration in YNB was tripled, there was inhibition of 3 and 4-spored ascus formation and an increase in total sporulation.

These results were investigated further by tripling the concentrations of individual trace elements and salts (Table 3). When added individually to YNBG PSM, none of the salts affected sporulation except calcium chloride and KH_2PO_4 which increased total sporulation but diminished 3 and 4-spored ascus yields. These two components of the salts solution can account for the effects described in Table 2. The zinc sulfate component of the trace elements solution, which diminished total sporulation but increased 3 and 4-spored ascus yields, can account for the effect shown in Table 2 when all the trace elements were tripled simultaneously in the PSM. The effect of individual vitamins remains to be studied.

Table 2

Effect of Tripling the Concentration of Groups of Constituents in Yeast Nitrogen Base (YNB) Growth Medium on Sporulation and 3 and 4-Spored Ascus Formation

Presporulation Medium	Total Sporulation,%**	3 and 4-Spored Asci,%**
6% glucose 0.67% YNB* (control)	67	16±3
6% glucose 0.67% YNB 4 ppm inositol 0.8 ppm calcium pantothenate 0.8 ppm niacin 0.8 ppm pyridoxine hydrochloride 0.8 ppm thiamine hydrochloride 0.004 ppm biotin 0.4 ppm para-aminobenzoic acid 0.004 ppm folic acid 0.4 ppm riboflavin	52	25±2
6% glucose 0.67% YNB 10,000 ppm ammonium sulfate	62	4±1
6% glucose 0.67% YNB 500 ppm $MgSO_4$ 150 ppm $CaCl_2.2H_2O$ 2,000 ppm KH_2PO_4 200 ppm NaCl	88	5±2
6% glucose 0.67% YNB 1 ppm Acid Boric 0.2 ppm KI 0.4 ppm $FeCl_3$ 0.8 ppm $ZnSO_4.7H_2O$ 0.4 ppm $Na_2MoO_4.2H_2O$ 0.8 ppm $MnSO_4$ 0.05 ppm $CuSO_4$	52	31±5

* The formulation of 0.67% YNB is given in Appendix I. The concentrations indicated in parts per million (ppm) were added to make the final concentrations equivalent to that of 2% YNB.

** Cells were grown 21 hr and after 48 hr in 3.5% potassium acetate sporulation medium, percentages of total sporulation and of 3 and 4-spored asci were determined by scoring 500 entities from each of 5 replicate flasks. The experiment was done twice.

Table 3

Effect of Tripling the Concentrations of Individual Salts* and Trace Elements in YNBG Presporulation Medium on Sporulation and 3 and 4-Spored Ascus Formation

Presporulation Medium	Total Sporulation,%**	3 and 4-Spored Asci,%**
6% glucose 0.67% YNB* (control)	77	16±3
Individual Salts Added:		
6% glucose 0.67% YNB 500 ppm $MgSO_4$	80	15±3
6% glucose 0.67% YNB 150 ppm $CaCl_2.2H_2O$	88	8±1
6% glucose 0.67% YNB 2,000 ppm KH_2PO_4	90	6±2
6% glucose 0.67% YNB 200 ppm NaCl	78	16±3

(continued on next page)

Table 3 (cont'd.)

Presporulation Medium	Total Sporulation,%**	3 and 4-Spored Asci,%**
Individual Trace Elements Added:		
6% glucose 0.67% YNB 0.05 ppm $CuSO_4$	78	16±2
6% glucose 0.67% YNB 0.4 ppm $Na_2MoO_4.2H_2O$	74	20±3
6% glucose 0.67% YNB 0.8 ppm $MnSO_4$	78	16±2
6% glucose 0.67% YNB 1 ppm Acid Boric	78	15±2
6% glucose 0.67% YNB 0.2 ppm KI	78	17±2
6% glucose 0.67% YNB 0.4 ppm $FeCl_3$	77	13±2
6% glucose 0.67% YNB 0.8 ppm $ZnSO_4.7H_2O$	63	31±5

* The formulation of 0.67% YNB is given in Appendix I. The concentrations indicated in parts per million (ppm) were added to make the final concentrations equivalent to that of 2% YNB.

** Cells were grown 21 hr and after 48 hr in 3.5% potassium acetate sporulation medium, percentages of total sporulation and of 3 and 4-spored asci were determined by scoring 500 entities from each of 5 replicate flasks. The experiment was done twice.

Effect of Varying the Zinc Sulfate Concentration During Growth and the Effect of Addition of Zinc Salts to Sporulation Medium

The results described above indicate that zinc is an important factor determining the manner of nuclear division during sporulation. Therefore, the effects of varying the concentration of zinc in the YNBG PSM were investigated, in an attempt to optimize the yield of 3 and 4-spored asci in potassium acetate SM.

Varying the concentration of zinc in YNBG PSM had a profound effect on the percentage of 3 and 4-spored asci formed in 3.5% potassium acetate SM (Figure 9). There was a broad optimum concentration range for 3 and 4-spored ascus production from 5 to 300 ppm zinc sulfate. The percentage of total sporulation was high at all concentrations of zinc sulfate supplied, but diminished appreciably above 125 ppm.

In order to test whether zinc could stimulate 3 and 4-spored ascus formation when added to SM instead of PSM, zinc acetate and zinc sulfate were added separately to 3.5% potassium acetate. The results are shown in the upper portion of Table 4. The addition of either zinc salt to the SM had no effect on total sporulation but gave a marked increase in the yield of 3 and 4-spored asci. This shows that cells can be conditioned to differentiate into 3 and 4-spored asci by adding zinc to SM.

In the lower portion of Table 4, the same zinc additions were made to SM but cells grown in PSM containing 25 ppm zinc sulfate were used. In comparing these results with those obtained using cells grown in PSM without added zinc sulfate, it is seen that the highest yields

Figure 9: Effect of addition of zinc sulfate to YNB presporulation medium supplemented with 6% glucose on sporulation and 3 and 4-spored ascus formation in 3.5% potassium acetate sporulation medium.

Appropriate aliquots from a stock solution consisting of 10,000 ppm zinc sulfate in deionized glass-distilled water were transferred to 300-ml volumes of 0.67% YNB supplemented with 6% glucose in deionized glass-distilled water, to give final concentrations of 0.5, 1, 5, 25, 125, 300 and 600 ppm zinc sulfate in this presporulation medium. Growth and sporulation media were inoculated and maintained as indicated in the preceding figures except that 3.5% potassium acetate was prepared in deionized glass-distilled water. The total sporulation (open circles) and yield of 3 and 4-spored asci (closed circles) were determined as previously described. The experiment was done twice. The graph is plotted on 4-cycle semi-logarithmic graph paper.

FIGURE 9

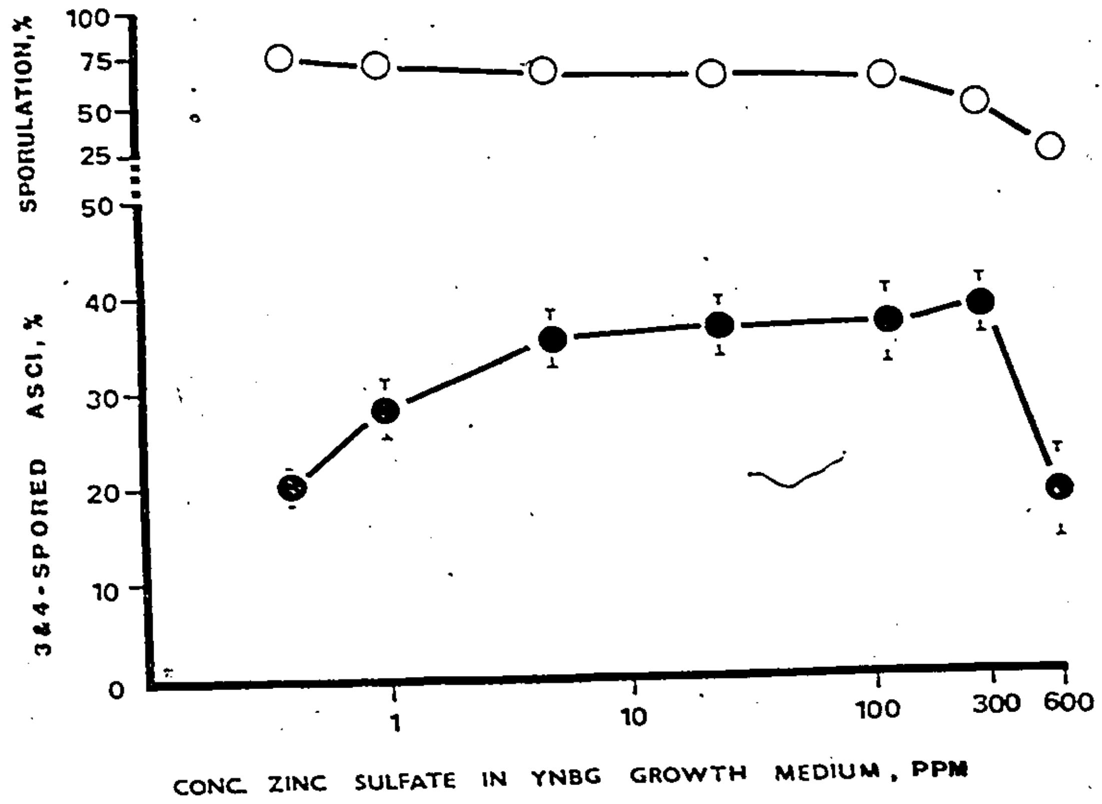


Table 4

Effect of Addition of Two Zinc Salts to Sporulation Medium on Sporulation and 3 and 4-Spored Ascus Formation

Presporulation Medium	Sporulation Medium**	Total Sporulation,%***	3 and 4-spored asci,%***
6% glucose 0.67% YNB* (control)	3.5% potassium acetate	73	20±2
6% glucose 0.67% YNB	3.5% potassium acetate 25 ppm zinc sulfate	73	33±3
6% glucose 0.67% YNB	3.5% potassium acetate 25 ppm zinc acetate	72	27±2
6% glucose 0.67% YNB 25 ppm zinc sulfate+ (control)	3.5% potassium acetate	61	36±3
6% glucose 0.67% YNB 25 ppm zinc sulfate+	3.5% potassium acetate 25 ppm zinc sulfate	65	48±2
6% glucose 0.67% YNB 25 ppm zinc sulfate+	3.5% potassium acetate 25 ppm zinc acetate	66	46±2

* The formulation of 0.67% YNB is given in Appendix I. This medium contains 0.4 ppm zinc sulfate.

** Sporulation media were sterilized by filtration.

*** Cells were grown 21 hr and after 48 hr in sporulation medium, percentages of total sporulation and of 3 and 4-spored asci were determined by scoring 500 entities from each of 5 replicate flasks. The experiment was done twice.

\+ Final concentration of zinc sulfate in presporulation medium.

of 3 and 4-spored asci were obtained when zinc was supplied in both media. 48±2% of the asci contained 3 or 4 ascospores when 25 ppm zinc sulfate was supplied in both the PSM and SM. This was the highest yield of such asci obtained at any time during this study.

Incidentally this experiment shows that zinc and not sulfate is the component of zinc sulfate which promotes 3 and 4-spored ascus development, since the same stimulation was obtained with both zinc acetate and zinc sulfate.

The Effect of Various Additions to YNBG Medium on Growth

In preceding experiments, a number of additions to YNBG PSM have been shown to diminish 3 and 4-spored ascus production: ammonium sulfate, the combined salts of YNB, "vitamin-free" casein hydrolysate, peptone and yeast extract (see Tables 1 and 2). It was of interest to relate their effects on growth to 3 and 4-spored ascus production. The growth data from the experiments are summarised in Table 5. The amendments all favoured growth, especially peptone. But in no case was growth as extensive as in MYPG medium. It is evident that their suppressing effect on 3 and 4-spored ascus production was not a result of restricting vegetative cell development in the growth medium.

As shown in Figure 10, increasing the glucose content in YNBG PSM decreased the cell population density achieved during 21 hr growth. As already indicated in Figure 3, cells grown at higher glucose concentrations were more competent to differentiate into 3 and 4-spored asci in SM.

The foregoing observations suggest that amendments which favoured growth inhibited 3 and 4-spored ascus formation in SM, and

Table 5

Effect of Factors Inhibiting 3 and 4-Spored Ascus Formation on Growth

Presporulation Medium	Cell Density at end of 21 hr growth** (millions/ml)
Basal medium*	47±5
Basal medium 10,000 ppm ammonium sulfate	63±3
Basal medium 500 ppm $MgSO_4$ 150 ppm $CaCl_2.2H_2O$ 2,000 ppm KH_2PO_4 200 ppm NaCl	76±6
Basal medium 0.1% casein hydrolysate ("vitamin-free")	77±5
Basal medium 0.5% peptone	120±5
Basal medium 0.3% yeast extract	97±7
MYPG consisting of 0.3% malt extract 0.3% yeast extract 0.5% peptone 0.1% KH_2PO_4 6% glucose	245±7

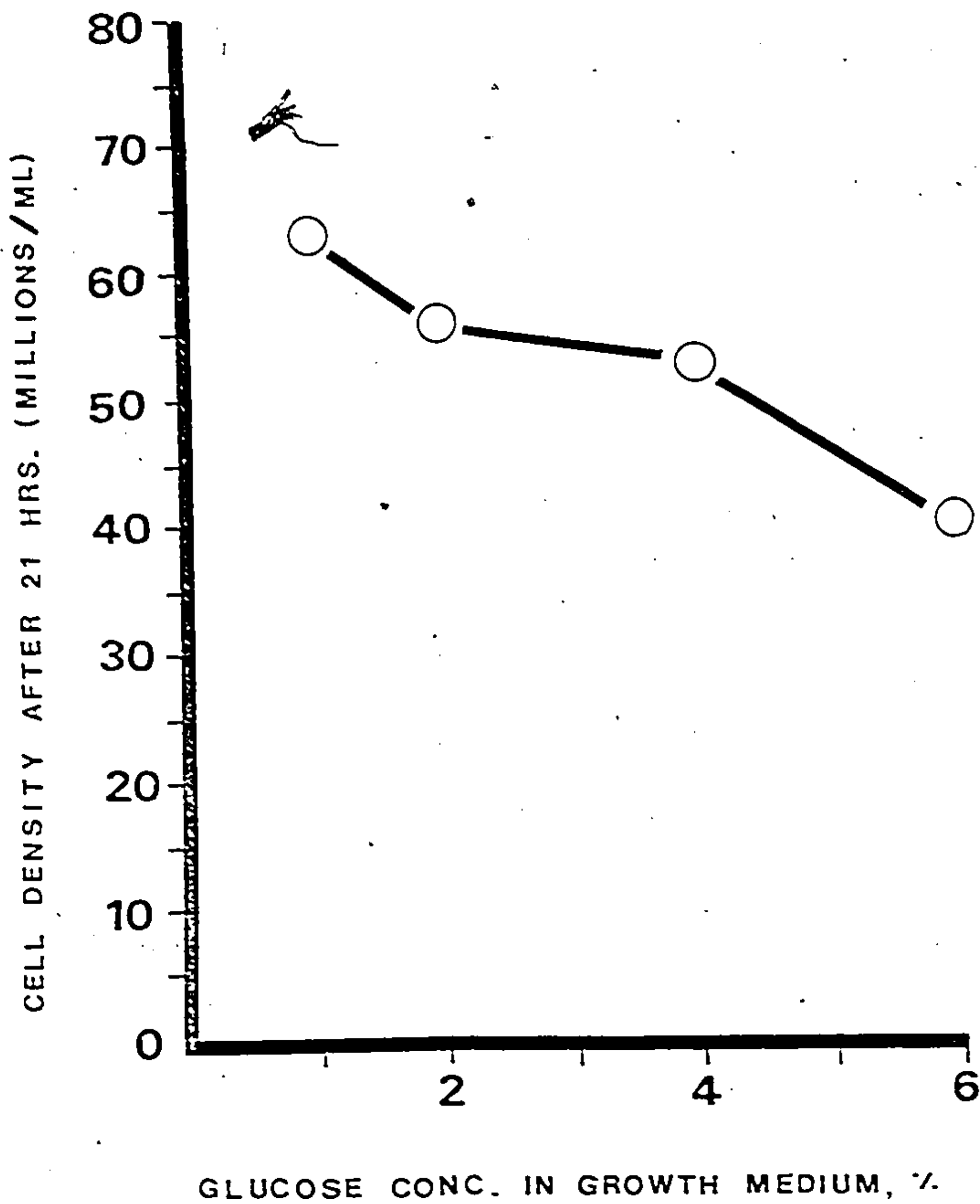
* Basal medium consisted of 0.67% YNB and 6% glucose.

** Samples were prepared for scoring with counting chambers by diluting a 1-ml sample of growth culture 100 times.

Figure 10: Effect of varying the glucose concentration in YNBG presporulation medium on cell density achieved during 21 hours incubation.

Growth cultures with initial cell densities of 10^5/ml in 50-ml 0.67% YNB supplemented with 1, 2, 4, and 6% glucose were incubated for 21 hr at 27°C in a water-bath shaker operated at 100 oscillations per min. A 1-ml aliquot was withdrawn from each growth culture and diluted to 1/100. The cell densities were determined by counting the number of cells in each of ten 0.1 mm^3 volumes using Spencer AO counting chambers.

FIGURE 10



that a condition which inhibited growth (high glucose concentration) had the opposite effect on such ascus production.

However, increasing the zinc sulfate content of YNB (Table 6) did not influence growth even at a concentration of 600 ppm. Frey *et al*. (1967) found that the concentration of zinc could be increased to 500 ppm with little inhibition of growth in brewing yeasts. Therefore, there appears to be no correlation between the effects of zinc on 3 and 4-spored ascus development and on growth.

Effect of Addition of Zinc to MYPG PSM on the Ability of Cells to Produce 3 and 4-Spored Asci in SM

Since zinc in YNBG PSM has a stimulating effect on 3 and 4-spored ascus production in SM, it was interesting to determine whether zinc additions to MYPG could restore 3 and 4-spored ascus production by cells grown on this medium. A concentration of 25 ppm zinc sulfate when added to this undefined medium was ineffective in promoting 3 and 4-spored ascus development in SM. However, 25 ppm zinc sulfate added to 3.5% potassium acetate SM had a slight stimulating effect on 3 and 4-spored ascus production by cells grown in MYPG: the percentage of such asci was increased from 0 (control value) to 1.5% by the addition of zinc sulfate to SM. When zinc was also present in the MYPG PSM, the percentage increase was from 0 to 1.4%.

Effect of Zinc on Growth in MYPG Medium

In the foregoing experiment, the concentration of zinc sulfate (25 ppm) supplied in MYPG could have been insufficient to promote 3 and 4-spored ascus formation during sporulation. To test this, concentrations above 25 ppm were added to the MYPG growth medium. When

Table 6

Effect on Growth of Varying the Concentration of Zinc Sulfate

Final Zinc Sulfate Concentration in YNBG basal medium* (ppm)	Cell density at end of 21 hours growth** (millions/ml)
0.4 (control)	52±5
1	56±12
5	51±10
25	49±7
125	47±12
300	47±12
600	51±8

* Basal medium consisted of 0.67% YNB and 6% glucose.

** Samples were prepared for scoring with counting chambers by diluting a 1-ml sample of growth culture 100 times.

concentrations of 125, 300, 600 and 1,000 ppm zinc sulfate were added to MYPG growth medium, precipitates formed. Perhaps these insoluble complexes resulted from an interaction between zinc and components of the MYPG medium. Similar precipitates formed when these concentrations of zinc sulfate were added to 0.3% yeast extract, 0.5% peptone and 0.1% "vitamin-free" casein hydrolysate. As shown in Table 1, the latter three substances diminished 3 and 4-spored ascus production when added to YNBG PSM at these concentrations. This suggested that the mechanism of this inhibition was the complexing of zinc with amino acids and possibly other compounds.

The zinc-treated MYPG media containing the precipitates were filter-sterilized and inoculated. Slight growth was observed with 125 ppm zinc sulfate and none at the higher concentrations of this substance. However, when 0.5% ammonium sulfate was also added to these media, abundant growth was observed after 21 hr incubation.

The restoration of growth in these media by the addition of ammonium, indicates that nitrogenous components of MYPG can bind to zinc, and therefore restrict the availability of this micronutrilite. Consequently, the cells are less able to produce 3 and 4-spored asci in SM.

Incidental Observations

(i) Regardless of the PSM (MYPG or YNBG) employed for cell growth, the 19e1 strain showed extensive flocculation when sporulated in 3.5% potassium acetate supplemented with 125, 300, and 600 ppm zinc acetate or zinc sulfate. Perhaps the zinc at these concentrations of zinc acetate or zinc sulfate in SM altered the surface charge

properties of the sporulating cells, causing them to adhere to each other.

(ii) Esposito and Esposito (1974) noted that buds detach from their parent cells before the latter sporulate in SM. When the 19e1 strain was grown in YNB supplemented with 6% glucose, and sporulated in 3.5% potassium acetate supplemented with 125 ppm zinc acetate or zinc sulfate, the detachment of buds from parent cells was inhibited. However, the inhibition of bud detachment was not observed when concentrations of 5 or 25 ppm were added to SM.

(iii) As mentioned previously, when the 19e1 strain was grown in YNB medium supplemented with 6% glucose, and sporulated in 3.5% potassium acetate supplemented with 25 ppm zinc acetate, approximately 50% of the sporulated cells differentiated into 3 and 4-spored asci. When asci harvested from this SM were resuspended in sterilized glass-distilled water and left at room temperature in the dark for 2 - 4 days, single ascospores, ascus wall material and 2-spored asci were observed under the light microscope. No 3 and 4-spored asci could be seen in the slides examined indicating that the free ascospores originated from such asci. Grewal and Miller (1972) noted that the ascospores in 2-spored asci in the 19e1 strain were connected by a conspicuous "intersporal body" which made ascospore separation difficult. The "intersporal bodies" were apparently absent in the 3 and 4-spored asci produced in the present study. Perhaps an enzyme system which hydrolyzes the carbohydrate polymers in the ascus walls was activated freeing the ascospores during the storage period. This

did not happen with the 2-spored asci because of the "intersporal body" connecting the two spores which prevents separation.

This observation might be useful for the genetic analysis of random spores provided that the 2-spored asci and non-sporulated cells could be separated from the ascospores derived from 3 and 4-spored asci.

DISCUSSION

Effect of Varying the Concentrations of Carbon Sources in Growth and in Sporulation Media on 3 and 4-Spored Ascus Development

As mentioned in the Introduction, there has been some study of the effect of the nature and concentration of carbon source supplied in PSM and SM on the number of spores per ascus. It is difficult to relate the work to that described here because the others studied normal four-spored strains. Here the yeast is normally 2-spored and its ascospore formation is preceded by a single nuclear division. Nevertheless there is some similarity in the results, in that with increasing concentration of carbon sources in PSM and SM the proportion of 3 and 4-spored asci increased. It should be stressed however that here not only the number of spores but also the number of nuclei per ascus were increased by raising the concentration.

The present study is moreover the first in which carbon source concentration has been varied in both PSM and SM. THe highest yields of 3 and 4-spored asci were obtained with 6% glucose in PSM and 3 to 3.5% potassium acetate in SM (Figure 3). With 4% glucose, and 2% glucose in PSM, the highest yields resulted at 3.5 and 4.5% potassium acetate respectively. When 1% glucose was added to YNB, the highest yields resulted at 4 to 4.5% potassium acetate. Thus, there was a tendency to shift from higher to lower optimum potassium acetate concentrations for 3 and 4-spored ascus production, as the glucose concentration added to YNB was increased.

A number of suggestions can be made to account for the stimulating effect of increased carbon source concentration in PSM and SM:

(i) According to Aldous et al. (1950), increasing the glucose concentration supplied in the growth medium increases cell fermentation activity and CO_2 production. Subsequently, Stoppani et al. (1958) reported that CO_2 fixation in yeast is an essential metabolic reaction, since CO_2 assimilation occurs during the oxidation of acetate. Bettleheim and Gay (1963) found that CO_2 assimilation is required for sporulation. In strain 19e1, it is possible that a biosynthetic pathway concerned with ascospore formation was stimulated by the intracellular level of CO_2.

(ii) Croes (1967 a,b) noted an increase in lipid and carbohydrate content in sporulating *Saccharomyces*. During sporulation, glycogen is synthesised (Roth, 1970), turned over (Hopper et al., 1974), and degraded during the period of ascospore formation (Colonna and Magee, 1978). Also, trehalose accumulates during yeast sporulation and it becomes localised in the ascospores (Roth, 1970). It is possible that the formation of 3 and 4-spored asci in strain 19e1, is related to the level of intracellular carbohydrate synthesised from the carbon sources supplied in PSM and SM.

(iii) Miller (1957) suggested that the oxygen consuming system plays an important role in determining the number of spores in an ascus. As the acetate concentration in SM is increased, one would expect more acetate to be assimilated by the cells via the TCA cycle. Such a mechanism might be involved in promoting nuclear division in

strain 19e1, and hence causing an increase in 3 and 4-spored ascus development.

The data allow no decision as to which of the foregoing explanations is most plausible to account for the formation of 3 and 4-spored asci in strain 19e1 and other explanations may also be possible.

Relationship of Other Nutrients in Growth Medium to the Number of Spores formed per Ascus in Sporulation Medium

When strain 19e1 was cultured in the undefined PSM malt extract-yeast extract-peptone-glucose (MYPG), the cells produced no 3 and 4-spored asci in potassium acetate SM, but when grown in the defined PSM (YNB + glucose), such asci were observed in SM. When the yeast extract and peptone components of MYPG were added to the defined growth medium, predominantly 2-spored asci resulted in SM. Casein hydrolysate had the same effect, indicating that free amino acids supplied during growth can diminish the ability to form 3 and 4-spored asci in SM. A single amino acid, tyrosine, also had this effect, but cystine had no effect.

The composition of Difco Yeast Nitrogen Base (YNB) is given in Appendix I. When the content of YNB was varied, without varying glucose (Figure 8), total sporulation increased to a fairly constant level, and the yields of 3 and 4-spored asci increased to an optimum at 0.5% YNB, above which yields declined. But some factor or factors when at higher concentration must have decreased the ability to form such asci. The further experiments in which groups of constituents, and

then individual constituents were varied in amount allow one to account for these trends.

$CaCl_2$ and KH_2PO_4 increased total ascus yields. A stimulating effect of calcium on ascus yields was reported by Kondratieva (1940), and McClary et al. (1959) found stimulation by potassium. The component of YNB that increased the yields of 3 and 4-spored asci was evidently $ZnSO_4$. The components which, when increased further, caused a reduction in yields of such asci were ammonium sulfate, $CaCl_2$ and KH_2PO_4. Ammonium when added to SM is well known to inhibit ascospore formation in normal 4-spored Saccharomyces, but the PSM must contain an adequate nitrogen supply to produce sporulation competent cells (Miller and Hoffmann-Ostenhof, 1964). Possibly excess ammonia favoured amino acid synthesis leading to high internal levels of amino acids. As noted above, amino acids added to YNBG PSM diminished 3 and 4-spored ascus production in SM.

The Effect of Zinc on Sporulation

Zinc appears to have a prominent role in the formation of 3 and 4-spored asci in SM, since the proportion of such asci was greatly increased when the zinc sulfate content in PSM was raised from 0.4 ppm to 1.2 ppm (Table 3). The highest concentration employed (600 ppm) inhibited the development of 3 and 4-spored asci (Figure 9), but had no effect on growth in PSM (Table 6). When zinc acetate or zinc sulfate was added to SM, there was a dramatic increase in the yield of 3 and 4-spored asci with no change in total ascus yields (Table 4). Thus, zinc and not sulfate, is responsible for promoting the formation of 3 and 4-spored asci in SM.

Could the addition of zinc to the undefined growth medium (MYPG) enable cells grown on this medium to form 3 and 4-spored asci in SM? Only when zinc was added to SM, were 3 and 4-spored asci obtained from cells grown in MYPG. As indicated in the Results, the assimilable nitrogen source precipitates out on the addition of zinc sulfate to MYPG. Dependent on the concentration of zinc sulfate added to MYPG, filtrates from zinc-treated MYPG media supported little or no growth unless supplemented with ammonium sulfate as the nitrogen source. Thus, the complexing of zinc with the assimilable nitrogen source in MYPG can account for both the absence of 3 and 4-spored asci in SM when cells were cultivated in zinc-treated MYPG media, and the marked decline in such ascus yields observed when cells were cultivated in the YNBG PSM amended with yeast extract, peptone, L-tyrosine or "vitamin-free" casein hydrolysate. Trace amounts of available zinc must be present in MYPG since this trace element is known to be essential for yeast growth (Olson and Johnson, 1948), but it is possible that zinc bound to certain amino acids in MYPG cannot be assimilated. It is of interest that some amino acids have been found to induce symptoms of zinc deficiency in mammals (Sadler, 1978).

According to Cochrane (1958), zinc has many diverse roles in fungal metabolism, and Reif <u>et al</u>. (1960) discuss its functions in yeast. Of interest in the present study is zinc's essentiality as a nutritional factor for vegetative growth and its involvement in the synthesis and activity of certain enzyme proteins. However, no attention has been given hitherto to the effect of this essential micronutrilite on yeast sporogenesis. The mode of action of zinc in

restoring normal ascus production in strain 19e1 is not known. Zinc-dependent enzymes are common in yeast and include: alcohol dehydrogenase (Vallee, 1955), fructose diphosphate aldolase (Rutter and Ling, 1958), lactic dehydrogenase (Gregolin and Singer, 1963) and D-glyceraldehyde-3-phosphate dehydrogenase (Keletti, 1964). Of particular interest is the association of zinc with proteolytic enzymes (Vallee, 1955; McConn _et al_., 1964; Tsuru _et al_., 1965). Sporulation specific proteinases are known in _Saccharomyces_ (Chen and Miller, 1968; Klar and Halvorson, 1975). Possibly an enzyme essential for normal ascus production in strain 19e1 requires unusually high intracellular levels of zinc to function in a process necessary for the nuclear divisions that precede the formation of 3 and 4-spored asci. It is noteworthy that Foster (1956) found that an excess of certain elements, e.g. potassium and manganese, supplied during growth are necessary to condition bacterial cells to subsequent sporulation. Another possibility is that the site of zinc action does not differ from normal 4-spored yeasts, but that strain 19e1 is unable to maintain the required intracellular zinc levels unless it is supplied in the medium.

In a study of seed production in peas and beans, Reed (1942) showed that definite threshold values of zinc appeared to exist, below which vegetative growth was good but seeds were not produced. In the presence of small quantities of zinc, growth was normal and pods developed, but they did not contain seeds. Increases in zinc supply gave striking increases in seed production with no effect on growth. Similarly in strain 19e1, increasing the zinc supply in YNBG PSM had no

effect on growth (Table 6), but gave striking increases in sexual spore production (Figure 9).

Cytological and Genetical Considerations

The cytological observations of nuclear divisions in developing asci indicate that nuclear division by meiosis precedes the formation of 3 and 4-spored asci in strain 19e1, and that a single equational nuclear division precedes the formation of 1 and 2-spored asci (Figure 6). In further support, conjugations were observed between cells from germinated ascospores derived from 3 and 4-spored asci (Figure 7), but not between those from 1 and 2-spored asci. But Ashraf and Miller (1978a) did not observe conjugation between cells from germinated ascospores originating from amitrole-induced multispored asci containing more than the usual 2 ascospores and this appears to conflict with the foregoing. However their asci frequently contained more than 4 spores and thus seemed abnormal, possibly owing to treatment with the herbicide.

Additional evidence indicates that nuclear division by meiosis precedes the formation of tetranucleate and hence 3 and 4-spored asci in strain 19e1:

(i) Glucose added to SM is known to block meiosis I in *Saccharomyces* (Miller, 1964) and this treatment gave a dramatic decrease in the yield of 3 and 4-spored asci in strain 19e1 (Figure 5).

(ii) When the 4 single-spore clones originating from each 4-spored ascus were cultivated on nutrient agar plates, a 2:2 segregation of pseudomycelial growth: normal growth was observed, and 2:1 and 1:2 segregations for these morphological characters were

observed when single-spore clones from 3-spored asci were cultivated in the same way.

With 2-spored asci, on the other hand, a single equational nuclear division precedes ascospore formation in SM for the following reasons:

(i) No conjugations were observed when spores in 2-spored asci germinated.

(ii) Single-spore clones derived from 2-spored asci were competent to sporulate.

(iii) Glucose added to SM did not inhibit 2-spored ascus production.

(iv) No segregation for the morphological marker was observed since spore clones derived from these asci showed growth indistinguishable from parental type growth, i.e. non-pseudomycelial growth was observed.

All single-spore cultures derived from 3 and 4-spored asci, however, were competent to sporulate and this may appear inconsistent with the foregoing conclusion. It seems that strain 19e1 is homothallic. In homothallic strains of S. cerevisiae, diploidization can occur through fusions between haploid cells of opposite mating type, both descendants of the same haploid ascospore. The phenomenon of self-diploidization is controlled by a set of dominant genes for homothallism designated HO, HM_a, HM_α (Winge and Roberts, 1949; Hawthorne, 1963; Takano and Oshima, 1969, 1970; Hicks and Herskowitz, 1976). The combination of the nonspecific HO gene with the specific HM_a gene directs the mutation of a to α during or soon after

germination of a single haploid ascospore of <u>a</u> mating type. The combination of HO and HM_{α} directs a mutation of <u>α</u> to <u>a</u> during or soon after the germination of a single haploid ascospore of <u>α</u> mating type. Harashima <u>et al</u>. (1974) reported that HO may direct a mutation of either mating type to opposite mating type when HM_a and HM_{α} are either both present or both absent. In view of this, it is possible that strain 19e1 is <u>a</u> HO HM_{α} HM_a/<u>α</u> HO HM_{α} HM_a or <u>a</u> HO hm_{α} hm_a/<u>α</u>HO hm_{α} hm_a. Within the first few mitotic divisions after the germination of single haploid ascospores from 3 and 4-spored asci derived from either parental genotype, self-mating between sister cells would be possible, and this may explain the observation of conjugation and the ability of single-spore clones to sporulate.

As discussed earlier, the assimilation of zinc, glucose, and acetate appear to be involved in the restoration of normal 3 and 4-spored ascus production in strain 19e1. Zinc is evidently an important determinant of reductional nuclear division during sporulation for the following two reasons:

(i) When cells were grown in YNB supplemented with 6% glucose and then sporulated in 3.5% potassium acetate, two classes of asci were observed: binucleate and tetranucleate asci (Figure 6). One and two-spored asci belonged to the former class, and 3 and 4-spored asci to the latter class. Under the same nutritional conditions employed for these observations, the addition of zinc in SM gave a marked increase in the yield of 3 and 4-spored asci without altering total ascus yields (Table 4). This indicates that the assimilation of zinc supplied in SM directs into reduction division cells which would have

undergone a single equational nuclear division in the absence of added zinc in SM.

(ii) No 3 and 4-spored asci were observed in SM when cells were grown in MYPG unless zinc was added to the SM.

Grewal and Miller (1972) were the first to report that the formation of two-spored asci in strain 19e1 is preceded by a single equational nuclear division. Such a yeast strain can be regarded as apomictic because it produces a sexual structure, the ascus, within which occurs an equational nuclear division that superficially resembles mitosis. Furthermore, no prior mating is required in order that cells may undergo the sporogenic process. Apparently, this is also the case in multispored strains of Lipomyces lipofer in which successive equational nuclear divisions precede ascospore formation (Henninger et al., 1974; Henninger and Emeis, 1974). Since the nutritional treatments employed with strain 19e1 appear to have restored meiotic nuclear division, it may be possible to take similar nutritional approaches with other apomictic organisms.

How did the nutritional manipulations with the zinc and glucose supply in PSM, and the zinc and acetate supply in SM restore normal reductional nuclear division in strain 19e1? Moens et al. (1977) observed rare synaptinemal complexes in sporulating cells of the apomictic yeast strain 4117, whose life cycle is similar to strain 19e1. However, when they force-mated 4117 with an adenine-requiring strain, and sporulated the progeny from the mated cells, ascospores from the resulting asci yielded diploid clones which gave the same two-spored character as the original 4117 strain in SM. Single-spore

clones derived from such asci retained the ability to sporulate without prior mating. However, unlike the original 4117 strain, these synthesised strains showed abundant synaptinemal complexes and meiotic levels of recombination during sporulation. On this basis, they suggested that the original strain 4117 retains a normal prophase and that following synapsis, recombination, and desynapsis, progressive development through meiosis I does not occur to completion, but instead meiosis II sets in with division of centromeres and chromosomes. Thus, binucleate asci result and the nuclei are diploid and of a/α mating type. It is possible that strain 19e1 behaves in this way during sporulation, and that the nutritional treatments employed in the present study restored the first reduction, i.e. the segregation of duplicated homologues at anaphase of meiosis I. With meiosis II and the segregation of sister chromatids, four haploid nuclei would result. However, the original 4117 strain showed rare synaptinemal complexes and in the author's opinion this may be due to transient pairing which is often observed in apomictic parthenogenetic insects, that produce diploid eggs from presumptive sexual cells which undergo a single equational nuclear division (Suomalainen et al., 1976). Hence, it is also possible that the apomictic yeast strains, 19e1 and 4117, are unusual asynaptic mutants, which undergo a single equational nuclear division during sporulation, as a result of the absence of pairing in the formation of synaptinemal complexes during prophase I. Since evidence in strain 19e1 indicates that reductional nuclear division precedes the formation of 3 and 4-spored asci, it would follow that normal synaptinemal complex formation occurs during prophase I. In the

absence of synaptinemal complex formation, it is conceivable that a single equational nuclear division occurs during sporulation. Since zinc appears to be a very important factor in the restoration of "normal" ascus production in strain 19e1, a zinc-related function might be involved in the required synaptinemal complex formation necessary for a normal meiosis. It would be interesting to know if meiotically competent and incompetent cells differ by the presence and absence of a fibrillar protein involved in the pairing of duplicated homologues, and whether a zinc-dependent protease modifies precursor proteins into active forms involved in such pairing. Perhaps zinc or a zinc protein is involved in the development of the synaptinemal complex.

Recently, Esposito's group found 2 genes for apomixis in strain 4117 (personal communication, M. Mowat). Perhaps similar genes for apomixis exist in strain 19e1, and are expressed in cells which undergo a single equational nuclear division during sporulation. One would expect the lack of expression of such genes in cells competent to undergo reduction division during sporulation, and perhaps zinc or a zinc-related function is involved in the regulation of the expression of these genes. In the past, there has been presented much evidence indicating the involvement of zinc in the regulation of protein synthesis in the fungi (Cochrane, 1958), and hence it is possible that zinc plays a similar role in strain 19e1. As there is lack of evidence, the foregoing is speculative. It is however testable and suggests feasible experiments.

In closing, there is a certain dualism in the life cycle of strain 19e1, since some cells appear to undergo reductional nuclear

division during sporulation instead of the usual equational nuclear division in response to nutritional treatments provided in the presporulation and sporulation phases. The alternative routes cells can follow during sporulation are shown in Figure 11. By manipulating the nutritional environment, some control of the manner of nuclear division has been achieved and this provides an excellent opportunity to investigate some biochemical and genetic controls of sexual reproduction.

Recommendations for Future Research on This Subject

Listed below are lines of investigation which follow logically from the work described above, and could be undertaken to expand the knowledge and control of yeast apomixis:

(i) Do the other two known apomictic strains of *S. cerevisiae* (ATCC-4117, ATCC-4098) respond similarly to the nutritional manipulations which promote the formation of 3 and 4-spored asci in strain 19e1?

(ii) Colonies derived from 3 and 4-spored asci of strain 19e1 showed segregation for a macromorphological marker, but presumably self-diploidization occurred and single-spore clones became competent to sporulate. Would subsequent generations be uniform for the morphological marker used?

(iii) What effect would other elements related to zinc have on sporogenesis?

(iv) There was some evidence that vitamins stimulated 3 and 4-spored ascus development and this could be investigated in more detail.

(v) What are the effects of other carbon sources, temperature, aeration, and pH on 3 and 4-spored ascus development?

(vi) Amitrole induces multispored ascus formation in strain 19e1. Would the effects of amitrole, high carbon source concentration, and zinc be additive?

(vii) Tyrosine added to the defined PSM inhibited subsequent 3 and 4-spored ascus development in SM but cystine did not at the concentrations employed. What would be the effect of other nitrogenous compounds on 3 and 4-spored ascus development?

(viii) Can typical Saccharomyces be made apomictic by environmental manipulation?

(ix) Would varying the time spent in the presporulation phase influence 3 and 4-spored ascus development in SM?

(x) Are there macromolecular differences between meiotically competent and incompetent cells of strain 19e1?

(xi) Can changes in fine structure, e.g. synaptinemal complex formation, be detected when 3 and 4-spored ascus production is restored?

Figure 11: Proposed alternative life cycles in *Saccharomyces cerevisiae* strain 19el.

1,2: Mitotic nuclear division of diploid nucleus during budding.

3: Mature bud ready to separate from mother cell. (Between 3 and 4 the yeast is transferred from growth to sporulation medium).

4: Diploid nuclei of some cells undergo reduction division yielding four haploid nuclei in the ascus, two of which are a mating type and two α mating type (left). Diploid nuclei of other cells divide equationally yielding two diploid nuclei of a/α mating type (right).

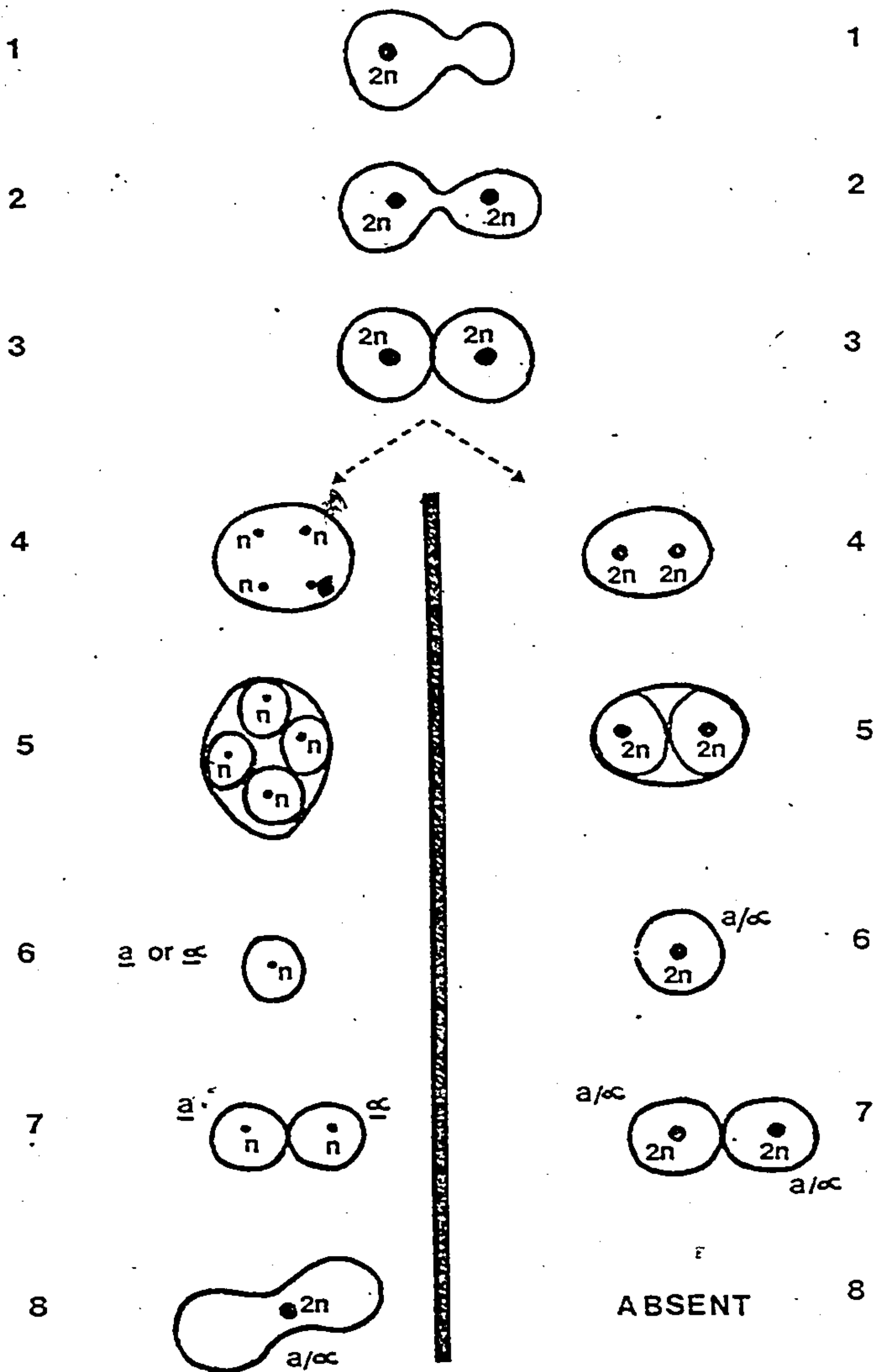
5: A spore wall forms around each nucleus.

6: A single spore isolated from the four-spored ascus (left) and a single spore isolated from the two-spored ascus (right) are transferred to germination medium.

7: During or shortly after the germination of the single haploid ascospore derived from the four-spored ascus, there is a directed mutation of the mating type allele to opposite mating type (left). This change of mating type does not occur with the ascospore derived from the two-spored ascus when it germinates since its nucleus is already a/α (right).

8: Plasmogamy followed by karyogamy between haploid sister cells of opposite mating type (left).

FIGURE 11



SUMMARY

1. A nutritional study was made of an apomictic strain of Saccharomyces cerevisiae which produced only two ascospores per ascus. By various treatments it was possible to enable this strain to form asci with three or four ascospores like typical strains of Saccharomyces cerevisiae. The following treatments favoured three and four-spored ascus development:

(a) cultivation in a medium lacking undefined components such as yeast extract, peptone and casein hydrolysate,

(b) increasing the glucose content of the defined medium from 1 to 6-8%,

(c) increasing the acetate concentration of the sporulation medium from 0.5 to 3-3.5%,

(d) increasing the zinc content of the defined presporulation medium and supplementing the sporulation medium with zinc, and

(e) adjusting the cell density in sporulation medium to 1-5 million/ml.

2. Total ascus yields were stimulated but three and four-spored ascus yields were diminished when the $CaCl_2$ and KH_2PO_4 concentrations in the defined presporulation medium were increased. When the ammonium sulfate concentration in the presporulation medium was increased, both the total sporulation and the yield of three and four-spored asci were diminished.

3. It was concluded that the induction of three and four-spored ascus formation was the result of a change from a single equational nuclear division to reductional nuclear division in the developing ascus for the following reasons:

(a) glucose added to sporulation medium inhibited such ascus formation,

(b) Giemsa staining demonstrated that two nuclear divisions preceded three and four-spored ascus formation,

(c) conjugations were observed during or shortly after ascospore germination, and

(d) segregation for a macromorphological marker was detected by microdissection followed by cultivation of ascospores from three and four-spored asci.

APPENDIX I

Composition of Yeast Nitrogen Base+

	Concentration in a 0.67% solution YNB (ppm)
Nitrogen source	
Ammonium sulfate	5,000
Amino Acids	
1-Histidine Monohydrochloride	10
dl-Methionine	20
dl-Tryptophane	20
Vitamins	
Inositol	2
Calcium Pantothenate	0.4
Niacin	0.4
Pyridoxine Hydrochloride	0.4
Thiamine Hydrochloride	0.4
Biotin	0.002
Para-Aminobenzoic Acid	0.2
Riboflavin	0.2
Folic Acid	0.002
Salts	
$MgSO_4$	250
$CaCl_2.2H_2O$	75
KH_2PO_4	1,000
NaCl	100
Compounds supplying trace elements	
Acid Boric	0.5
KI	0.1
$FeCl_3$	0.2
$ZnSO_4.7H_2O$	0.4
$Na_2MoO_4.2H_2O$	0.2
$MnSO_4$	0.4
$CuSO_4$	0.025

+ Wickerham (1951)

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