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Induction of Normal Ascosporogenesis in Two-Spored *Saccharomyces cerevisiae* by Glucose, Acetate, and Zinc

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In an apomictic strain of *Saccharomyces cerevisiae* that normally produces two-spored asci, production of three and four-spored asci was restored by adjusting zinc and carbon source levels in presporulation and sporulation media. Sporogenic capability was compared after growth in defined and undefined presporulation environments. Cultivation in defined presporulation medium containing 6% glucose followed by sporulation in 3.5% acetate yielded 16 ± 3% three- and four-spored asci. Inclusion of 25 μg of zinc sulfate per ml in presporulation medium or 25 μg of zinc acetate per ml in sporulation medium gave a further increase in yield of three- and four-spored asci; it reached 48 ± 1% when zinc was added to both media. Cells grown in undefined presporulation medium were incapable of three- and four-spored ascus development in acetate sporulation medium unless zinc was included in the latter medium. Cultivation in defined presporulation medium amended with yeast extract, peptone, or casein hydrolysate greatly reduced three- and four-spored ascus yields. Inclusion of a meiotic inhibitor (glucose) in sporulation medium inhibited three- and four-spored ascus production but not two-spored ascus production. Nuclear staining demonstrated two classes of asci: binucleate (one and two spored) and tetranucleate (three and four spored). Hence, some control over apomictic versus normal ascus development has been gained by nutritional manipulation.

Of the 15 strains of *Saccharomyces cerevisiae* examined by Grewal and Miller (6) which formed few or no asci with more than two spores, 12 apparently underwent normal meiosis during sporulation since the asci always contained four nuclei. Three were unique in that only one nuclear division occurred per ascus. They reported that these strains (19e1, ATCC 4117, ATCC 4098) were unlike typical *S. cerevisiae* in that: (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 spore was apparently diploid; (iv) conjugations were never observed during spore germination; (v) clones derived from single germinated spores were competent to sporulate; and (vi) sporulation was not glucose inhibited. They assumed that the nuclear division in the asci was mitotic. In an electron microscope study of strains 19e1 and ATCC 4117, Moens (9) reported that the single nuclear division could be distinguished from mitosis by the presence of polycomplex bodies known to contain synaptonemal complex-like elements, the nuclear and spindle pole body behavior, and the loss of the parental nucleolus in the epiplasm surrounding the spores. Recently, a combined genetical and cytological study (10) of strain 4117 suggested that a normal meiotic prophase was retained during sporulation and that after synapsis, recombination, and desynapsis, the asci did not complete meiosis I, but, instead, proceeded to meiosis II, with segregation of chromatids.

Such yeasts are apomictic (1) because a sexual structure (ascus) is produced within which gamete formation is bypassed and the resultant progeny give rise to clones that can sporulate without prior mating.

It is well known that alterations in nutritional and other cultural conditions can influence the sporogenic capability of *S. cerevisiae* (4, 7). In this investigation we report the apparent restoration of three- and four-spored ascus development in an apomictic yeast (*S. cerevisiae* strain 19e1) by manipulation of nutritional conditions in presporulation and sporulation phases.

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MATERIALS AND METHODS

Organism and maintenance. Stock cultures of *S. cerevisiae* strain 19e1 were maintained on slants of Wickerham (17) yeast nitrogen base (YNB) containing 2% glucose and 2% agar (Difco).

Presporulation phase. Cells for inoculation of

sporulation cultures were cultivated in either of two types of presporulation medium (PSM). (i) A defined YNB medium supplemented with glucose as the carbon source (YNBG) was prepared directly in final strength by dissolving 0.67 g of YNB (Difco) in 100 ml of deionized, glass-distilled water containing the desired concentration of glucose; (ii) an undefined medium, malt extract-yeast extract-peptone-glucose (MYPG), was prepared as previously described (17) except that 0.1% KH_2PO_4 was included. The defined medium was sterilized by filtration, and the undefined medium was sterilized by autoclaving. Modifications of these media were made according to experimental requirements. Volumes (50 ml) of PSM in 250-ml Erlenmeyer flasks were inoculated with cells at an initial density of 10^5/ml. Cell numbers were estimated with Spencer AO hemocytometer counting chambers. Growth culture flasks were incubated 21 h at 27°C in a Warner-Chilcott Laboratories model 2156 water bath shaker operated at 100 oscillations per min.

Sporulation phase. Cells were harvested from PSM by centrifugation, washed twice with sterile, deionized, glass-distilled water, and dispensed in 50-ml volumes of potassium acetate sporulation medium (SPM) in 250-ml flasks at a cell density of 10^7/ml. The flasks were incubated in the same manner as growth cultures, and sporulation percentages were determined after 48 h. The mean percentage of sporulated cells and the percentage of the total asci that were three and four spored were determined with counting chambers by scoring 500 entities from each of five replicate flasks. All experiments were done in duplicate.

Chemicals. The following analytical grade reagents were obtained from the J.T. Baker Chemical Co.: anhydrous dextrose, potassium acetate, $ZnSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, and $(NH_4)_2SO_4$. The following analytical grade chemicals were obtained from the Fisher Scientific Co.: H_3BO_3, $MgSO_4$, $MnSO_4 \cdot H_2O$, $CuSO_4$, KH_2PO_4, NaCl, $Na_2MoO_4 \cdot 2H_2O$, $CaCl_2 \cdot 2H_2O$, KI, $Zn(C_2H_3O_2) \cdot 2H_2O$. "Vitamin-free" casein hydrolysate was supplied by Nutritional Biochemicals Corp. (Cleveland, Ohio).

RESULTS

Effect of varying the carbon source concentration in presporulation and sporulation media on sporulation. The effects of varying the concentrations of glucose supplied in YNB PSM and of acetate in SPM on total ascus yields are shown in Fig. 1A. At acetate concentrations of 2% and less, there was little effect of glucose concentration in PSM on total sporulation. But above 2% acetate, there was a decrease in yields, with increasing concentration of glucose in PSM.

The highest yields of three- and four-spored asci were obtained with 6% glucose in YNB PSM and 3 or 3.5% acetate in SPM (Fig. 1B). With 2 and 4% glucose in PSM, highest yields were at 4.5 and 3.5% acetate, respectively, i.e., as the glucose concentration in PSM increased, the optimum acetate concentration for three- and four-spored ascus production was lowered. The

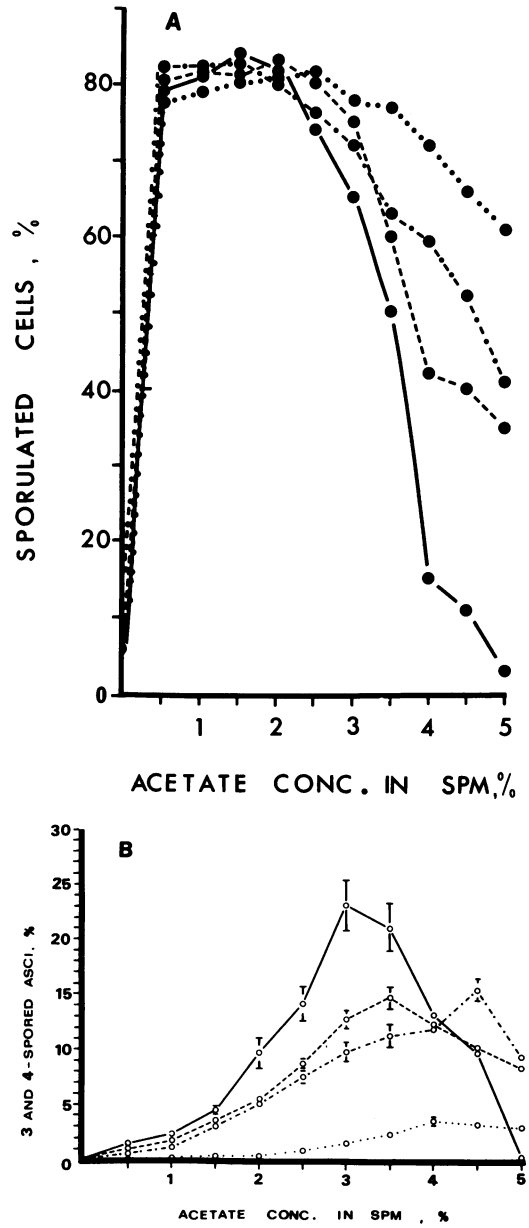

FIG. 1. *Effect of varying the carbon source concentration in PSM and SPM on ascus production. Glucose concentrations supplied in YNBG PSM: 1% (···), 2% (- · -), 4% (- - -), and 6% (——). (A) Percent total sporulation (●). (B) Percent three- and four-spored asci (○). Vertical bars indicate standard deviation.*

experiment was repeated in part, substituting the undefined MYPG medium for YNBG as the PSM. One, two, and six percent glucose were included in MYPG and cells were transferred to 2, 3, 3.5, and 5% acetate for sporulation. Asci

with more than two spores were not observed. Nutritional factors other than carbon source concentration must therefore affect three- and four-spored ascus production. Cell density appeared unimportant, since varying this parameter from 10^6 to 20×10^6/ml in 2% potassium acetate SPM was found to have little, if any, effect on three- and four-spored ascus yields.

Effect of an inhibitor of meiosis on sporulation. Glucose, even at 0.13%, strongly inhibited three- and four-spored ascus production, but not total sporulation in 2% acetate SPM (Fig. 2).

Effect of adding MYPG components and casein hydrolysate to YNBG presporulation medium. Cultivation of cells in YNBG plus malt extract or KH_2PO_4 (at same concentration as in MYPG) had no apparent effect on yields of three- and four-spored asci, but yeast extract and peptone markedly reduced the yields (Table 1). Casein hydrolysate also reduced the yields.

Effect of components of the defined YNB medium on three- and four-spored ascus production. Varying the YNB content of PSM (i.e., all other constituents except glucose) gave a maximum percentage of three- and four-spored asci in SPM at 0.5% YNB (Fig. 3). Since total

TABLE 1. *Effect of addition of MYPG components and vitamin-free casein hydrolysate to YNBG PSM on sporulation and three- and four-spored ascus formation*[a]

Additions to presporulation medium[b] (6% glucose, 0.67% YNB)	Total sporulation (%)	Three- and four-spored asci (%)
Control	86.8 ± 1.9	4.9 ± 0.5
0.1% KH_2PO_4	87.2 ± 1.8	4.0 ± 0.4
0.3% Malt extract	87.6 ± 1.3	4.4 ± 1.0
0.5% Peptone	94.4 ± 0.8	0.6 ± 0.2
0.3% Yeast extract	86.5 ± 1.4	0.1 ± 0.2
0.1% Casein hydrolysate (vitamin-free)	86.0 ± 2.1	0.3 ± 0.2

[a] Each value recorded represents the mean and standard deviation.

[b] After 21 h of incubation in PSM cells were harvested and transferred to 2% acetate SPM.

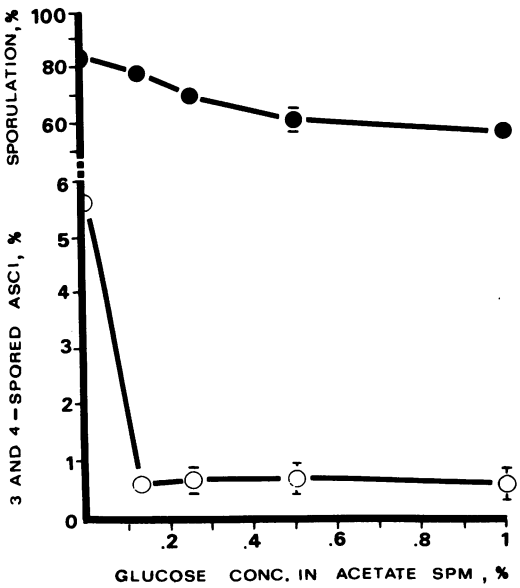

FIG. 2. *Effect of addition of glucose to acetate SPM on total sporulation and on yield of three- and four-spored asci. After 21 h of incubation in 50 ml of YNB PSM containing 6% glucose, cells were harvested and transferred to 50-ml volumes of 2% acetate SPM containing 0, 0.125, 0.25, 0.5, and 1% glucose. Percent total sporulation (●), percent 3- and 4-spored asci (○). Vertical bars indicate standard deviation.*

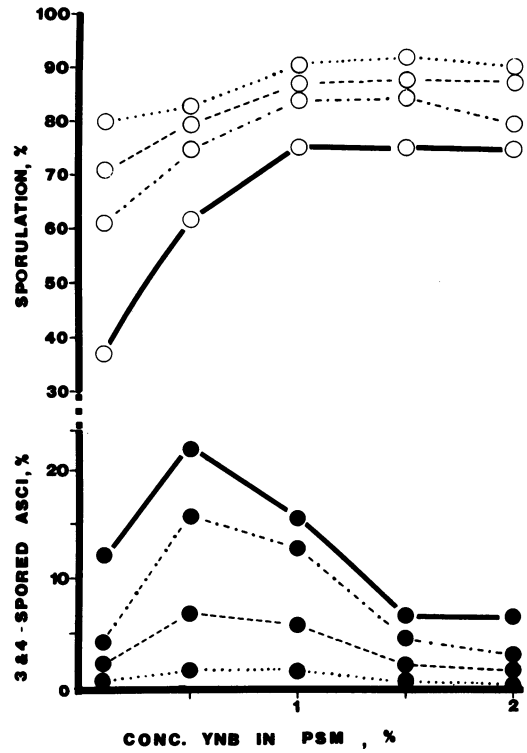

FIG. 3. *Effect of varying YNB content of PSM containing 6% glucose on % total sporulation and percent three- and four-spored asci in varying acetate concentrations. Cells were inoculated into 50-ml volumes of solutions, containing 0.1, 0.5, 1, 1.5, and 2% YNB in 6% glucose. Cells from each PSM were harvested, and the appropriate volume of cell suspension in each case was transferred to 50 ml of acetate SPM of known concentration. 1% (···), 2% (– – –), 3% (·–·–·), 3.5% (——). Percent total sporulation (○), percent 3- and 4-spored asci (●).*

sporulation was high after growth at the highest YNB concentration supplied, physiological conditions for sporulation were evidently maintained even at the highest YNB concentration (2%). The percentage of three- and four-spored asci, however, decreased at the higher concentrations. These changes in percentage of three- and four-spored asci indicate the presence of stimulating and inhibitory factors in the YNB medium.

When the content of individual constituents (except glucose) in YNBG PSM was tripled (Table 2), $CaCl_2$ and KH_2PO_4 increased total sporulation but decreased three- and four-spored ascus yields. Ammonium sulfate slightly decreased total sporulation and markedly decreased three- and four-spored ascus yields. Zinc sulfate slightly diminished total sporulation, but it approximately doubled the yield of three- and four-spored asci.

Effect of varying the zinc sulfate concentration supplied during growth and the effect of two zinc salts added to sporulation medium. The foregoing indicated that the zinc content of the PSM is an important factor determining the number of spores per ascus in this yeast strain. When the zinc sulfate content of the YNBG PSM was varied (Fig. 4), a broad optimum concentration range for three- and four-spored ascus production was evident. The percentage of total sporulation was fairly high at all zinc sulfate concentrations supplied, but diminished appreciably above 125 μg/ml.

TABLE 2. *Effect of tripling the concentrations of individual components of YNBG PSM on sporulation and three- and four-spored ascus formation in 3.5% potassium acetate SPM*[a]

Additions to presporulation medium[b] (6% glucose, 0.67% YNB)	Total sporulation (%)	Three- and four-spored asci (%)
None (control)	78.6 ± 2.0	16.0 ± 2.5
$(NH_4)_2SO_4$ (10,000)	61.5 ± 6.5	3.5 ± 0.7
$MgSO_4$ (500)	79.9 ± 4.3	15.0 ± 3.3
$CaCl_2 \cdot 2H_2O$ (150)	88.3 ± 1.0	8.5 ± 1.2
KH_2PO_4 (2,000)	89.7 ± 1.8	6.4 ± 1.8
NaCl (200)	78.2 ± 3.3	14.4 ± 2.1
$CuSO_4$ (0.05)	78.1 ± 4.0	15.7 ± 2.5
$Na_2MoO_4 \cdot 2H_2O$ (0.4)	74.1 ± 4.0	20.3 ± 2.5
$MnSO_4 \cdot H_2O$ (0.8)	78.4 ± 1.6	16.0 ± 2.0
H_3BO_3 (1)	78.4 ± 1.7	14.6 ± 1.8
KI (0.2)	78.7 ± 1.8	17.2 ± 1.6
$FeCl_3 \cdot 6H_2O$ (0.4)	77.6 ± 2.5	13.4 ± 2.3
$ZnSO_4 \cdot 7H_2O$ (0.8)	63.3 ± 6.6	31.2 ± 5.2

[a] Each value recorded represents the mean and standard deviation.

[b] The concentrations indicated in parentheses in micrograms per milliliter were added to make the final concentrations equivalent to that of 2% YNB.

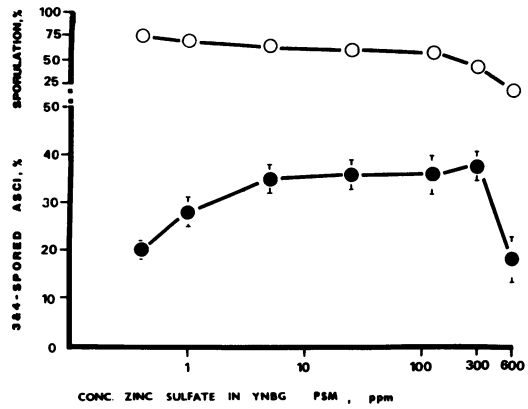

FIG. 4. *Effect of addition of zinc sulfate to YNBG PSM on sporulation and three- and four-spored ascus formation in 3.5% acetate SPM. Appropriate aliquots from a stock solution consisting of 10,000 μg of zinc sulfate per ml in deionized, glass-distilled water were transferred to 0.67% YNBG PSM containing 6% glucose in deionized glass-distilled water, to give final concentrations of 0.5, 1, 5, 25, 125, 300, and 600 μg of zinc sulfate per ml. Percent total sporulation* (○), *percent three- and four-spored asci* (●). *The graph is plotted on semilogarithmic paper. Vertical bars indicate standard deviation.*

Zinc also had a stimulating effect when added to SPM. As shown in the upper portion of Table 3, addition of either zinc acetate or zinc sulfate to SPM did not alter total ascus yields, but markedly increased the yield of three- and four-spored asci. In the lower portion of Table 3, the same zinc additions were made to SPM, but cells grown in PSM containing 25 μg of zinc sulfate per ml were used. It is seen that the highest yields resulted when zinc was supplied in both PSM and SPM.

As noted above, MYPG-grown cells did not form asci with more than two spores in 3.5% acetate SPM. However, it was found that inclusion of 25 μg of zinc acetate per ml in SPM enabled MYPG-grown cells to yield 1.4 ± 1% three- and four-spored asci.

Effect of zinc on growth. No significant difference was observed between cell densities achieved after 21 h in YNBG (6% glucose) PSM containing 0.4, 1.2, 5.0, 25, 125, 300, or 600 μg of zinc sulfate per ml. With MYPG PSM, however, addition of 300 or 600 μg of zinc sulfate per ml caused a precipitate to form. Filtrates of the medium thus treated did not support cell multiplication unless supplemented with ammonium sulfate. Filtrates from medium treated with 25 or 125 μg of zinc sulfate per ml supported less growth than untreated controls.

Cytological observations. From examination of many thousands of asci with a Giemsa

TABLE 3. *Effect of addition of two zinc salts to SPM on sporulation and on three- and four-spored ascus formation*[a]

Presporulation medium	Additions to 3.5% potassium acetate sporulation medium[b]	Total sporulation (%)	Three- and four-spored asci (%)
6% Glucose–0.67% YNB[c]	None (control)	74.1 ± 1.6	20.1 ± 1.9
6% Glucose–0.67% YNB[c]	Zinc sulfate (25)	73.1 ± 2.7	32.3 ± 4.1
6% Glucose–0.67% YNB[c]	Zinc acetate (25)	72.0 ± 1.2	27.3 ± 1.2
6% Glucose–0.67% YNB–25 of zinc sulfate[d] per ml	None (control)	61.3 ± 1.5	34.5 ± 2.4
6% Glucose–0.67% YNB–25 of zinc sulfate[d] per ml	Zinc sulfate (25)	64.8 ± 3.6	48.0 ± 1.1
6% Glucose–0.67% YNB–25 of zinc sulfate[d] per ml	Zinc acetate (25)	66.3 ± 2.3	45.9 ± 2.3

[a] Each value recorded represents the mean and standard deviation.
[b] Sporulation media were sterilized by filtration. Concentration in parentheses was micrograms per milliliter.
[c] The control contained 0.4 μg of zinc sulfate per ml.
[d] Final concentration of zinc sulfate in PSM.

staining procedure (13), we determined that asci in strain 19e1 contained either two nuclei (one- and two-spored asci) or four nuclei (three- and four-spored asci). Three-spored asci contained an unenclosed nucleus, as observed by previous workers (11, 12). One-spored asci contained one unenclosed nucleus and two-spored asci contained no unenclosed nuclei.

DISCUSSION

That nutritional adjustment influences the number of spores per ascus in *Saccharomyces* is evident from previous investigations (4, 7) with yeasts in which ascospore formation is preceded by meiosis. However, our treatments, which increased the number of spores per ascus, apparently did so by restoring normal meiotic nuclear division. This is also indicated by the fact that with an inhibitor of yeast meiosis, glucose (8) in the SPM, only two-spored asci were formed.

It is evident that the production of three- and four-spored asci is affected by zinc levels supplied in PSM and SPM. The proportion of such asci was greatly increased by raising the zinc content of the PSM (Table 2, Fig. 4) and zinc additions to SPM increased yields still further (Table 3). Zinc supplied in SPM did not affect total sporulation but conditioned to undergo two nuclear divisions, cells which would have undergone only one nuclear division in its absence.

Increasing the level of compounds supplying other micronutrilites in PSM did not stimulate three- and four-spored ascus development (Table 2). In fact, two salts known to inhibit zinc uptake during yeast growth, $CaCl_2$ and KH_2PO_4 (3, 5), inhibited three- and four-spored ascus production.

Although adjusting carbon source concentrations of PSM and SPM allowed three- and four-spored ascus production by cells grown in YNB PSM (Fig. 1), no carbon source concentration was found to allow this when using the undefined PSM. Zinc added to SPM, however, did enable the development of a few three- and four-spored asci after cultivation under the undefined conditions. Evidence indicated that amino acids (in casein hydrolysate) and possibly other nitrogenous compounds bind zinc, restricting its availability during vegetative growth to yield cells capable of only two-spored ascus development. It is noteworthy that some amino acids may produce zinc deficiency symptoms (15).

The stimulation of three- and four-spored ascus production by increasing carbon source levels in PSM may be related to an enhancement of zinc uptake (3). Zinc has been reported to affect nucleic acid stability (18), spindle stability (2), and the activity of certain enzyme proteins (16). Previous work (9, 10) with a similar apomictic strain (ATCC 4117) indicated that the binucleate and, hence, two-spored condition results from cells undergoing meiosis II without the reductional chromosome segregation of meiosis I. Possibly strain 19e1 is unable to retain zinc levels required for processes related to the segregation of duplicated homologs at anaphase of meiosis I unless it is supplied exogenously. A similar situation has been found with peas and beans, in which zinc levels exceeding those required for normal vegetative growth are necessary for meiosis and, hence, seed formation (14).

Some control over apomixis has evidently been gained in this yeast, providing an opportunity to investigate further mechanisms controlling apomixis and meiosis.

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