EFFECT OF POTASSIUM VERSUS SODIUM IN THE SPORULATION OF SACCHAROMYCES[1]

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Adams (1949, 1950) described a modified Stantial (1935) acetate medium consisting of low concentrations of glucose, sodium acetate, and agar upon which he obtained high yields of asci with a large number of yeast cultures. Although, in his original experiments, Adams (1949) tested a variety of acetate salts, including potassium acetate, he found none of them superior to sodium acetate in about 0.24 per cent concentration.

These reports have been followed by a rather extensive series of papers in which Adams' sodium acetate medium has been used with only slight modifications. These include the hybridization and genetic studies of Fowell (1952, 1955) and the extensive studies of physiological factors affecting yeast sporulation on sodium acetate medium of Adams and Miller (1954); Miller *et al.* (1957*a*, *b*); Scheiber *et al.* (1957); Tremaine and Miller (1954, 1956); Miller and Halpern (1956); and Miller (1957).

The sodium acetate medium has also proved very useful in cytological studies of McClary *et al.* (1957*a*, *b*) and of Hashimoto *et al.* (1958).

Although many Lindegren cultures of Saccharomyces sporulated abundantly on sodium acetate-yeast extract-glucose medium, others produced asci so rarely that they could not be used in genetic studies. The evolution of the Lindegren breeding stocks are described in the papers of Lindegren and Lindegren (1943*a*, *b*). They determined that genetic analysis depended upon the isolation of haploid strains which did not produce spores unless mated to other haploid strains of complementary mating type. Continued selection of cultures resulted in a breeding stock with this characteristic, although early in the pedigree, sporulation among single ascospore segregants was not too unusual. It was presumed that much of this was due to illegitimate copulation producing illegitimate diploids, but some of the haploid segregants appeared, on occasion, to produce spores. Whatever the cause, this character was bred out of the stock, and single ascospore segregants which produced spores became very rare. Conversely, diploid hybrids between two cultures of complementary mating type did not invariably produce ascospores. Sporulation among diploids varied from those of very low frequency to others of very high frequency, and a wide range of "fertility" (ability to sporulate) of cultures was characteristic. This was found especially true of hybrids heterozygous for many hereditary characteristics. A culture freshly isolated from nature might sporulate abundantly, but continued selection of mutants often led to the production of strains which sporulated poorly. This situation was characteristic not only of yeasts but also of Neurospora (Lindegren *et al.*, 1939). One may, therefore, expect to find a wide range of fertility, some diploids or polyploids sporulating rarely, or not at all on a certain medium, and others sporulating abundantly. This large, heterogeneous group of yeasts, with respect to sporulation, maintained in this laboratory, presented an opportunity for extensive work on the problem of sporulation in Saccharomyces.

To test the effect of different substrates upon sporulation, it is necessary to consider the effect of the substrate on poorly sporulating as well as vigorously sporulating strains. Evidence that an agent effects sporulation is more significant when the effect is shown to be active against cultures which normally sporulate very poorly and less significant if the culture is one which sporulates abundantly under almost any circumstance. A sporulation medium consisting of 0.1 M potassium acetate, 0.25 per cent yeast extract, and 0.1 per cent glucose in distilled water has been formulated which significantly increases yields of asci in several Lindegren cultures, as well as some commercial strains of yeasts, over those obtained in sodium acetate medium. The experiments upon which these modifications of the original sodium

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acetate medium are based are the subject of this paper.

MATERIALS AND METHODS

Cultures. Lindegren diploid, triploid, and tetraploid cultures of Saccharomyces were used as indicated in each experiment. Some of these cultures were aerobic deficient, i. e., incapable of oxidative respiration on glucose and failing to produce the alkaline reaction to phenol red in the peptone-acetate-phenol red medium of Ogur *et al.* (1954). These characteristics are indicated when applicable to the experiments. Stock cultures were maintained on agar slants of the presporulation medium described below with 2 per cent agar added. The cultures were covered with sterile mineral oil and kept in the refrigerator to prevent sporulation of some of the cells with a consequent change in ploidy and other genetic characteristics.

Basal media. The presporulation medium contained glucose, 20 g; $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 2 g; yeast extract, 5 g; and distilled water to 1000 ml.

The basal medium for sporulation consisted of anhydrous sodium acetate, 8.2 g; glucose, 1.0 g; yeast extract,[2] 2.5 g; and distilled water to 1000 ml.

These media were modified as indicated in each experiment and compared with control experiments using the above, unmodified media.

Quantitative methods. Cells from the stock cultures were grown in 20 ml of presporulation broth in 250-ml Erlenmeyer flasks on a shaker at 28 C for 2 days, washed twice with sterile, distilled water by centrifugation and decanting, and resuspended to their original volume with distilled water. One-tenth ml of the cell suspension was transferred to 10 ml of the sporulation medium in a 250-ml Erlenmeyer flask, giving a final concentration of approximately 3 million cells per ml—a concentration within the range found by Miller *et al.* (1955) to be optimal for spore formation and with which our results have agreed. The use of a shallow layer of broth on a shaker insures a more uniform suspension of cells than does the solid medium recommended by Adams, and it is believed to be better for quantitative methods of determining the formation of asci. This technique is based upon the procedure used by Foster (1956) in the production of endospores by bacteria. Counts were made of vegetative cells and asci, usually after 3 days, and the percentages of asci were calculated.

[2] Yeast extract (Difco), liquid yeast extract (Anheuser-Busch), and basamin (Anheuser-Busch) all produce essentially the same results.

EXPERIMENTS AND RESULTS

Optimum acetate concentration. Adams (1949, 1950) recommended 0.14 per cent anhydrous sodium acetate in his sporulation medium. Figure 1 shows the effect of increasing concentrations of sodium acetate in yeast extract-glucose medium. Calculated as anhydrous sodium acetate, the optimal concentration in this medium, for the yeast culture used, was between 0.6 and 0.84 per cent, or about 0.1 M.

Effects of mineral salts. The stimulating effects of several metallic ions upon bacterial sporulation, particularly Mg^{++}, Mn^{++}, Ca^{++}, and K^{+}, have been reported by numerous authors, much of whose work has been reviewed by Curran (1957). Much less has been reported concerning the effects of mineral salts upon ascospore production in yeasts.

Welten (1914) reported the enhancement of sporulation in yeasts by magnesium sulfate. Oberfeuchtner-Gruber in 1949 (cited by Oppenoorth, 1957) described the stimulating effects of potassium salts. McClary and Nulty (1957) reported the increase in yields of asci in a sodium acetate-yeast extract-glucose medium when potassium fluoride and magnesium or calcium was added. Adams (1949) did not find potassium acetate or magnesium acetate any more stimulating in the sporulation of his yeast cultures than sodium acetate, and concluded that the stimulating effect of these salts was due entirely to the acetate ion.

The effects of increasing concentrations of potassium chloride, magnesium sulfate, calcium chloride, and manganese sulfate in the sodium acetate basal medium are presented in table 1. Potassium chloride in 0.025 M concentration effected almost a 4-fold increase in the yield of asci over the control. Magnesium sulfate, at concentrations between 0.002 and 0.0029 M exerted a slightly stimulating effect, but was inhibitory at higher concentrations. It is evident that magnesium can be supplied sufficiently in the presporulation medium to satisfy its requirement in the sporulation medium. Calcium chloride and manganese sulfate could not be shown stimulating at any concentrations used, but were inhibitory at the higher concentrations studied.

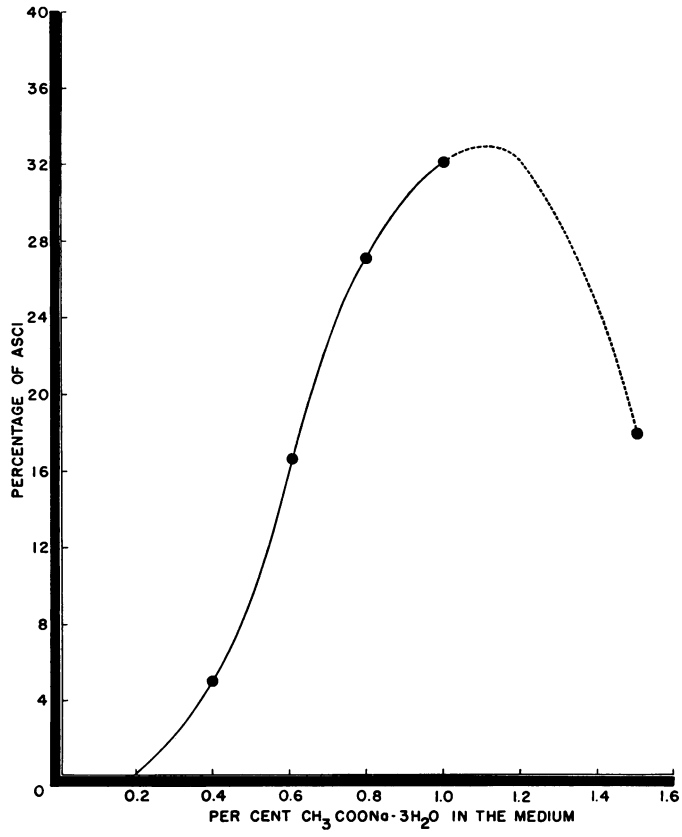

Figure 1. Effects of increasing concentrations of $CH_3COONa\text{-}3H_2O$ upon yields of asci by Saccharomyces culture 14268 × 8256 in yeast extract-glucose medium.

In table 2, the yields of asci obtained by several cultures of yeasts in the basal medium to which was added 0.025 M potassium chloride are compared with those obtained in the basal medium to which no potassium was added. It is seen that 10 of the 15 cultures produced increased yields—some actually producing quite prolifically on the potassium fortified medium, and producing no asci on the basal medium. In only one culture was there found to be a slightly higher yield of asci in the absence of potassium, and that was so slight as to be considered insignificant. The two cultures which did not produce asci on either medium were aerobic deficient. It is not known why the other two produced such low yields.

To determine whether or not sodium exerted an influence upon sporulation, a medium was prepared using the same molar concentration of potassium acetate as that of sodium acetate used in the basal medium, and increments of sodium chloride from 0.0167 to 0.025 M were added. It is shown in table 3 that the potassium acetate medium produced almost a 4-fold higher yield of asci than did the sodium acetate medium, and that the addition of sodium chloride produced no significant effect upon the yield when added to the potassium acetate medium.

The comparison of yields of asci from several cultures of Saccharomyces on sodium acetate and potassium acetate media is shown in table 4. For all except one, in which there was no significant difference, the potassium acetate was a superior sporulation medium.

Comparison of yeast extract-enriched-acetate medium, Adams' sodium acetate-glucose medium, and acetate media fortified with various other complex organic substances. In table 5, the yields of asci on several different media are compared. Adams' medium consisting of 0.14 per cent

TABLE 1

Effects of potassium chloride, calcium chloride, magnesium sulfate, and manganese sulfate on sporulation of Saccharomyces, diploid culture 14268 × 8256

Additions to Sodium Acetate-Yeast Extract-Glucose Basal Medium	Conc	Asci after 72 Hr
	M	%
Control: no additions	—	21
Potassium chloride	0.0167	52
	0.020	65
	0.025	74
	0.033	74
Magnesium sulfate	0.002	23
a. Cells from complete presporulation medium	0.0029	38
	0.004	17
	0.005	11
	0.010	5
b. Cells from presporulation medium less magnesium sulfate	—	11
	0.002	23
	0.0029	23
	0.004	14
Calcium chloride	0.002	21
	0.004	21
	0.0067	17
	0.010	16
Manganese sulfate	0.0005	22
	0.00067	18
	0.0010	16
	0.0020	0

sodium acetate and 0.04 per cent glucose produced a significantly higher yield of asci when fortified with potassium and magnesium, but its yield was not equal to that obtained in potassium acetate-yeast extract-glucose medium. Trypticase and beef extract were almost as effective as yeast extract in the potassium acetate-fortified medium, but proteose peptone and peptone (Difco) depressed yields lower than that obtained on the mineral-fortified Adams' medium. Efforts to increase the yields of spores from these media by removing possible inhibitors with activated charcoal, as accomplished by Foster (1956) for the sporulation of bacilli, were unsuccessful. Vitamin-free casein hydrolyzate almost completely inhibited sporulation in the sodium acetate-dextrose medium, and it was only slightly improved when the vitamins necessary for vegetative growth of the yeast were added.

TABLE 2

Effects of potassium chloride on yields of asci by 15 cultures of Saccharomyces in sodium acetate-yeast extract-glucose medium

Yeast Strain	Asci in Basal Medium	Asci with 0.025 M KCl Added	Degree of Ploidy	Respiratory Ability*
	%	%		
14268 × 8256	26	79	Diploid	AER
F-1 (Fleischmann's)	24	52	Probably diploid	AER
14720 × 14262	22	49	Diploid	AER
15191 × 15476	<1	<1	Diploid	AER?
14716 × 8256	20	42	Diploid	AER
15189 × 15789	<1	<1	Diploid	AER?
11296 × 10446	41	76	Triploid	AER
8324 × 8282	16	30	Diploid	AER
13778 × 15791	41	47	Diploid	AER
11294 × 13778	30	89	Triploid	AER
15477 × 14061	<1	19	Diploid	AER
20877 × 20059	11	48	Diploid	AER
20877 × 20080	22	48	Diploid	AER
19583 × 18854	0	0	Diploid	aer
18430 × 19583	0	0	Diploid	aer

* AER = respiratory sufficient on acetate; AER? = slight aerobic positive test; and aer = aerobic negative.

TABLE 3

Comparative yields of asci by Saccharomyces culture 14268 × 8256 in sodium acetate medium and potassium acetate medium fortified with NaCl and $MgSO_4$

Media	Additions to Media	Asci
		%
Basal sodium acetate medium	—	19
Potassium acetate medium	—	67
	0.020 M NaCl	71
	0.0167 M NaCl and 0.0029 M $MgSO_4$	70
	0.020 M NaCl and 0.0029 M $MgSO_4$	77
	0.0167 M NaCl and 0.0029 M $MgSO_4$	76

TABLE 4

Yields of asci of 12 different cultures of Saccharomyces on sodium acetate and potassium acetate sporulation media

Yeast Culture	Asci in Sodium Acetate Medium	Asci in Potassium Acetate Medium
	%	%
14268 × 8256*	26	79
14268 × 8256*	21	74
14268 × 8256*	26	63
14268 × 8256*	19	76
14268 × 8256*	27	65
11294 × 11296	85	76
15477 × 14061	2	43
11294 × 13778	70	86
15389 × 15789	1.4	28
13778 × 15791	30	75
14716 × 8256	29	64
14720 × 14262	18	64
8324 × 8252	25	70
11296 × 10446	55	69
8256 × 5.2M	0	0
Saccharomyces ellipsoideus	0	38

* Several comparative results are presented with this culture since it was the one used in most of the experiments.

TABLE 5

*Yields of asci by Saccharomyces culture 14268 × 8256 on various sporulation media**

Sporulation Medium	Asci
	%
Adams' sodium acetate-glucose medium less agar	30
Adams' medium plus 0.0031 M $MgSO_4$ and 0.025 M KCl	49
Sodium acetate-yeast extract-glucose medium	30
Basal medium plus 0.0029 M $MgSO_4$ and 0.025 M KCl	65
Trypticase medium plus 0.0029 M $MgSO_4$ and 0.025 M KCl	56
Proteose peptone plus 0.0029 M $MgSO_4$ and 0.025 M KCl	21
Bacto peptone plus 0.0029 M $MgSO_4$ and 0.025 M KCl	26
Beef extract (Bacto) plus 0.0029 M $MgSO_4$ and 0.025 M KCl	47

* Trypticase, proteose peptone, Bacto peptone, and beef extract were substituted in the basal medium for yeast extract.

DISCUSSION

In comparing the results obtained with several strains of yeasts on sporulation media containing potassium acetate with those containing sodium acetate, the following facts are evident: (a) Potassium itself is required for sporulation of some strains of yeast in higher quantities than is generally expected, and this requirement exists regardless of its concentration in the presporulation medium. (b) Although sodium acetate produces, in some vigorously sporulating strains of yeasts, yields of asci equal to those obtained in potassium acetate medium, it is not superior to potassium for these strains. (c) Sodium itself has no apparent stimulating effect upon sporulation and becomes inhibitory to some yeast strains in less than one-quarter the concentration of potassium when both are used as acetates. The last of these comparisons is derived from table II of Adams' article (1949).

Efforts to correlate the effect of potassium with physiological factors which might indicate its role in the sporulation process were not revealing. Alten and Rathje (1952), in studying the effects of potassium on respiring yeast cells, found that the potassium content of the cells paralleled their intensity of respiration, and concluded that potassium ions were exchanged for hydrogen ions in the respiring cells. Warburg respirometer determinations during this study, however, revealed no significant difference in the oxygen uptake of sporulating yeast cells in sodium acetate and potassium acetate medium. Neither were there differences in pH changes in sporulating cultures in potassium acetate and sodium acetate media taken at intervals for 4 days. Each rose steadily from pH 6.7 at the beginning to pH 9.9 at which time maximal spore counts were obtained.

Other cations, particularly Mg^{++}, Ca^{++}, and Mn^{++}, are not required in the sporulation medium for cells which were grown in presporulation media containing sufficient quantities of these elements for optimal growth. Acetate salts of these elements cannot be used successfully to supply the acetate for sporulation because of their inhibitory effect at concentrations of the acetate ion which are required. These results are in agreement with those of Adams (1949).

Although the simple acetate-glucose medium of Stantial (1935) and Adams (1949) has the advantages of a synthetic medium for certain physiological studies, it is inadequate for sporula-

tion of some strains of yeasts for cytological and genetic studies in which high yields of spores are required. One effect of the complex medium, in fact, may be the selective reproduction of a larger proportion of respiratory sufficient cells which are able to produce spores.

No attempt has been made to review all of the factors affecting ascospore formation in yeasts. Some of the more extensive reviews dealing with this subject are those of Lindegren and Lindegren (1944), Phaff and Mrak (1948, 1949), and the numerous articles of the Canadian workers (Adams, Miller, and their co-workers) cited in the introduction of this article. The simple media which will induce high degrees of sporulation in some strains of yeasts indicates that most of the important factors lie in the presporulation phase, and that the sporulation phase may be analogous to the intracellular protein degradation and resynthesis hypothesis postulated by Hardwick and Foster (1952) for spore maturation in the bacteria. The essential difference is the requirement of yeast for acetate or, perhaps, some other source of energy which can be oxidized only through the aerobic respiratory mechanism. Bacteria studied by Foster and Hardwick sporulated prolifically in distilled water.

The potassium acetate-yeast extract-glucose medium, though perhaps not satisfying the requirements of certain strains of yeast as well as others, has proved superior to other media tested in both genetic and cytological studies with the large collection of the Lindegren strains. By adding 2 to 3 per cent agar, it is made very convenient for a variety of genetic studies which require hybridization and sporulation. Since mating and sporulation both occur readily on this medium, many different genetic strains of asci can be obtained from several mating stocks on one agar plate by mixing compatible mating pairs of cells in spots on the agar. One plate will usually yield mature asci of 15 to 20 hybrids by this method in 2 or 3 days.

SUMMARY

Slight modifications have been made in the sodium acetate-glucose medium of Adams which have significantly increased yields of asci in several strains of Saccharomyces. KCl and magnesium sulfate, when added in certain concentrations to sodium acetate-yeast extract-glucose medium, have more than doubled the yields of asci in most cultures of yeasts tested. Potassium acetate may be substituted for sodium acetate in the sporulation medium with correspondingly high results.

Although considerable sporulation occurred in Adams' sodium acetate-glucose medium, it was increased significantly by the addition of magnesium, potassium, and yeast extract. The large amount of debris in the absence of yeast extract and the increased sporulation in its presence may indicate the necessity of exogenous sources of nutrients in the sporulation phase, other than acetate, regardless of the nutritional characteristics of the presporulation phase.

Practical applications of this medium solidified with agar have been suggested.

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