Growth Requirements of *Schizosaccharomyces octosporus*, a Yeast Exacting towards Adenine

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SUMMARY: The growth requirements of a strain of *Schizosaccharomyces octosporus* were studied by auxanographic and tube tests. The organism grows satisfactorily on a glucose + mineral salts medium containing asparagine and ammonium sulphate, provided also that inositol, pantothenic acid, nicotinic acid, biotin, histidine, methionine and adenine are added. Complex interactions between adenine and guanine were found, and for this reason *S. octosporus* cannot be regarded as a suitable test organism for the assay of adenine.

The present investigation arose from studies still in progress of the inositol requirements of a number of yeasts. Twelve strains were examined, and growth of eleven of them was satisfactory when inositol or yeast extract was added to a defined medium containing glucose + mineral salts, ammonium sulphate, casein hydrolysate and B vitamins. The exception was *S. octosporus* which failed to grow in absence or presence of inositol. Further investigations showed that *S. octosporus* required adenine, not hitherto reported as an essential metabolite for yeasts, and its growth requirements were therefore studied in more detail.

METHODS

Tube tests. The usual procedure for microbiological assay was followed, but in place of bacteriological tubes, flat-bottomed tubes (Samco), $4 \times \frac{3}{4}$ in., were used. Tubes were selected so that the internal diameter was within the limits 1.66 and 1.72 cm. The tubes, containing 6 ml. of test medium and covered with loosely fitting glass caps, were sterilized for 10 min. at 10 lb. pressure. After inoculation, they were incubated in a vertical position at 25° in a water-bath.

Estimation of amount of yeast growth. The tubes were fitted into a black wooden container open at opposite sides which held them in the optical beam of the Spekker absorptiometer, in the place normally occupied by the glass cells. Thus turbidity readings could be taken from the same tube at intervals during the growth of the organism, without exposing the contents to contamination.

Test organism and inoculum. The test organism, carried on malt agar slopes, was subcultured weekly or more frequently as required. In the preparation of the inoculum a loopful of a 24 hr. culture was suspended in 6 ml. of basal medium to give a turbidity in the tubes reading between 0.15 and 0.25 in the Spekker (i.e. approximately 0.2-0.4 mg. dry weight yeast/6 ml.). For inoculation one drop (20 μ l.) of the suspension was added to each tube from a sterile Pasteur pipette.

Auxanographic tests. See Pontecorvo (1949); Cutts & Rainbow (1949).

Media. Basal medium I. Composition per 500 ml. final volume was, in g.:

glucose, 10.0; L-asparagine, 1.0; KH_2PO_4 , 0.75; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; $(\text{NH}_4)_2\text{SO}_4$, 1.0; in $\mu\text{g.}$: aneurin HCl, 250; pyridoxin HCl, 250; Ca D-pantothenate, 250; nicotinic acid, 250; D-biotin, 0.1; KI, 50; in ml.: trace element solution (Emery, McCleod & Robinson, 1946), 0.5; lactate buffer, 5.0. The lactate buffer was prepared by adjusting 2.5 ml. lactic acid (syrupy A.R.) to pH 5.0 with KOH and making up to 5 ml.

Basal medium II. As for medium I with the addition of: inositol, 3.33 mg.; DL-methionine 8.33 mg.; L-histidine HCl, 4.17 mg./500 ml. of medium.

Basal medium III. As for medium I, with the addition of 1 g. casein hydrolysate/500 ml. of medium, the weight being based on the amino-N content ($\times 6.25$) of a solution prepared from Glaxo vitamin-free casein (Barton-Wright, 1946).

RESULTS

Tube tests with twelve yeasts. In these experiments basal medium I was used. Series of tubes were set up containing basal medium only, basal medium + 6.7 $\mu\text{g.}$ inositol/ml., and basal medium + 1 mg. Difco Bacto yeast extract/ml. After sterilization and cooling, the various sets were inoculated, and duplicate tubes read in the Spekker at intervals of 22, 30, 47, 72 and 120 hr. Table 1 summarizes the results obtained at 72 hr.

Table 1. *The effect of inositol and yeast extract in basal medium I on the growth of twelve yeasts*

Organism	Basal medium I with		
	No addition	Inositol 6.7 $\mu\text{g.}/\text{ml.}$	Yeast extract 1 mg./ml.
	Spekker readings at 72 hr.		
<i>Kloeckera brevis</i>	0	1.21	1.37
<i>K. apiculata</i>	0	1.19	1.40
<i>Torula utilis major</i>	1.18	1.23	1.28
<i>Saccharomyces carlsbergensis</i>	1.40	1.51	1.58
<i>S. intermedius</i>	1.00	1.39	1.58
<i>S. exiguus</i>	1.49	1.49	1.65
<i>S. validus</i>	0.09	0.74	1.55
<i>S. turbidans</i>	1.36	1.43	1.63
<i>S. pastorianus</i>	0.43	1.40	1.63
Bottom yeast	0.23	0.87	1.00
<i>Schizosaccharomyces pombe</i>	0	1.25	1.40
<i>Schizosacc. octosporus</i>	0	0	0.90

Of all the yeasts studied only *S. octosporus* failed to grow in the basal medium alone and in the presence of inositol. The extent of growth in the basal medium + yeast extract plainly indicated the presence in the latter of substances essential for this yeast, and which were lacking in the basal medium. By comparison with more complex liquid media employed in microbiological assays, for example, the deficiencies appeared likely to be amino-acids or purine-pyrimidine bases.

Auxanographic tests with S. octosporus. In a preliminary survey the following substances in various combinations were added to auxanographic plates:

casein hydrolysate, inositol, *p*-aminobenzoic acid, riboflavin, folic acid, adenine, guanine and uracil. It was clear from the results of these experiments that growth took place only when casein hydrolysate, inositol and adenine were added together. Accordingly, the requirements for adenine and amino-acids were examined.

Effect of purine-pyrimidine bases. Series of tubes were prepared with basal medium III (6 ml.) + 6.7 µg. inositol/ml. of medium. Sets were also made up to contain in addition to the foregoing, in µg./ml. of medium: (i) 4.2, 8.3, 16.7 adenine; (ii) 8.3 each of adenine, guanine and uracil; (iii) 8.3 each of guanine and uracil; (iv) 1000 Difco Bacto yeast extract. After sterilization, the tubes were inoculated with a 24 hr. inoculum of *S. octosporus* and the turbidities measured at intervals, with the results shown in Table 2.

Table 2. *The effect of purine-pyrimidine bases in basal medium III on the growth of Schizosaccharomyces octosporus*

(Supplements indicated as follows: I=inositol; A=adenine; G=guanine; U=uracil; YE=Difco Bacto yeast extract. Amounts in µg./ml. medium indicated by numbers.)

Basal medium III plus	Period of incubation, hrs.				
	48	55	74	96	142
	Spekker readings				
6.7 I	0	0	0	0	0
6.7 I + 4.2 A	0.31	0.47	0.64	0.74	0.85
6.7 I + 8.3 A	0.34	0.58	0.87	1.01	1.12
6.7 I + 16.7 A	0.35	0.60	1.10	1.24	1.29
6.7 I + 8.3 each of A, G, U	0.34	0.55	1.00	1.19	1.27
6.7 I + 8.3 each of G, U	0	0	0.02	0.05	0.53
6.7 I + 1000 YE	0.63	0.80	1.02	1.13	1.17

The marked stimulation of growth due to adenine was clearly shown, there being no growth in the absence of purines and pyrimidines. With guanine and uracil together growth proceeded fairly rapidly after a 96 hr. lag, while with 8.3 µg. each of adenine, guanine and uracil growth equivalent approximately to 16.7 µg. adenine was obtained. Yeast extract increased the rate of growth in the early stages; but it seemed to contain insufficient purines and pyrimidines for optimal growth of the organism.

Effects of purine and pyrimidine bases in admixture. These experiments suggested the investigation of the separate and combined effects of the purines adenine, guanine and xanthine, and the pyrimidines uracil, thymine and cytosine. Casein hydrolysate could be satisfactorily replaced by methionine and histidine (see below), and accordingly basal medium II was used in the experiment. Purines and pyrimidines in various combinations were added at the rate of 16.7 µg./ml. of medium to sets of tubes which respectively contained: (i) no added adenine; (ii) 0.83 µg. adenine/ml.; (iii) 33.3 µg. adenine/ml. of medium. The following effects were observed: (a) good growth took place after 96 hr. in presence of guanine and absence of adenine; (b) guanine (16.7 µg./ml.) exerted a strong inhibitory effect on growth in presence of 0.83 µg. adenine/ml.,

particularly during the first 96 hr.; (c) xanthine in presence of 0.83 μ g. adenine/ml. had a stimulatory effect on growth.

Further investigation of the interrelations of adenine and guanine revealed that the effects observed were dependent on the quantitative relationships of the two bases. At suboptimal levels of adenine, the effect of increasing amounts of guanine was a stimulation which reached a maximum when the molar ratio of guanine to adenine was about 2:1. At this ratio, the effect of guanine was equivalent to that of a similar quantity of adenine. For example, 2 μ g. guanine + 1 μ g. adenine produced a response equal to that of 3 μ g. adenine. When the ratio of guanine to adenine increased beyond 2:1 inhibition occurred, and at a molar ratio of 10:1 inhibition was almost complete. These results are shown in Fig. 1.

Fig. 1

Fig. 1. Interaction of adenine and guanine. Figures on curves indicate amounts of added adenine μ g./ml.

Fig. 2

Fig. 2. Comparison of effects of adenine and adenylic acid. Ad = with adenine, Ay = with adenylic acid. Ratio of molecular weights of adenylic acid and adenine is approximately 2.5.

With mixtures of xanthine and adenine, no inhibitory effect due to xanthine was revealed, and relatively large amounts of xanthine were required for stimulation. Thus 150 μ g. xanthine when added to 5 μ g. adenine produced growth equivalent to that obtained with about 10 μ g. adenine.

Effect of adenylic acid. Adenylic acid was found to replace adenine, but it was less effective on a molar basis, particularly at the lower dosage levels (Fig. 2).

Amino-acid requirements of S. octosporus. The amino-acid requirements of the organism were determined qualitatively by auxanographic tests which indicated clearly the essential nature of methionine and histidine. Tube-test experiments were then carried out which showed that in absence of both amino-acids no visible growth took place within 90 hr., and was only just

observable at 140 hr. With $33.3 \mu\text{g.}$ methionine/ml., growth commenced at 70 hr., and with the same amount of histidine growth was observed at 50 hr., and reached a slightly higher limit than that attained with 2 mg. casein hydrolysate/ml. (Fig. 3). The apparent lack of adequate amounts of histidine and methionine in the casein hydrolysate could not be compensated by increasing the amount of casein hydrolysate in the medium. On the contrary, increasing amounts of casein hydrolysate involved a certain degree of inhibition of growth. This may be due to the presence of increasing amounts of arginine and lysine which were observed to have a marked inhibitory effect both in auxanographic and tube experiments.

Fig. 3. Effect of methionine and histidine. *A*, without amino-acids; *B*, with methionine; *C*, with histidine; *D*, with methionine and histidine. Each amino-acid added at level of $33.3 \mu\text{g./ml.}$

Vitamin requirements of S. octosporus. Tube tests were used to examine the vitamin requirements, with a basal medium III containing $16.7 \mu\text{g.}$ adenine/ml., and all the vitamins B except that which was being studied. The organism required inositol, pantothenic acid, nicotinic acid and biotin; pyridoxin, *p*-aminobenzoic acid, riboflavin and folic acid did not appear to be necessary, and aneurin had a slight stimulatory effect. The addition of 1 mg. Difco Bacto yeast extract/ml. to basal medium III containing $16.7 \mu\text{g.}$ adenine/ml. and $6.7 \mu\text{g.}$ inositol/ml. produced only a very slight increase in growth, suggesting that this medium is essentially complete.

DISCUSSION

The fact that the strain of *S. octosporus* examined can grow normally only in the presence of adenine suggests that this organism is deficient in its ability to synthesize adenine from simpler constituents. Guanine is also required, and if not already present in the medium may be readily produced by conversion of the equivalent amount of adenine. Support for this suggestion is derived from the following observations: (*a*) in the presence of guanine alone the organism can grow only after a lag phase of some 4 days, presumably as a result of a slow conversion of guanine to adenine; (*b*) but when guanine is added to a

suboptimal concentration of adenine growth is improved to an extent similar to that which would have been obtained by addition of an equivalent amount of adenine, provided that the molar ratio of guanine to adenine does not exceed 2:1.

In the study of a *Neurospora* mutant requiring adenine, Fairley & Loring (1949) concluded that guanine was synthesized from adenine, and pointed out that the biosynthesis of guanine is analogous to that reported by Brown, Roll, Plentl & Cavalieri (1948) for the white rat. In this case, experiments with labelled adenine and guanine showed that whereas adenine could be converted to guanine, the reverse change was not effected. Our results with *S. octosporus* show that this strain is able to achieve the conversion in both directions, and apparently differs in this respect from the *Neurospora* of Fairley & Loring. Although these workers produced no evidence that their organism could utilize guanine, this might have been forthcoming had growth been studied over a longer period.

It has been shown that maximum stimulation and inhibition by guanine depends on the ratio guanine : adenine over a wide range of concentrations and not on the absolute values. It seems possible, therefore, that they, or their metabolic products, are competing for an enzyme system.

A strain of *S. cerevisiae*, designated 'Yeast 47', reported by Cutts & Rainbow (1949), can grow in the absence of *p*-aminobenzoic acid when adenine, methionine and histidine are supplied. They suggested that *p*-aminobenzoic acid may form part of a co-enzyme involved in the synthesis of these substances. While *S. octosporus* is not exacting towards *p*-aminobenzoic acid (and there is evidence that it can synthesize it), it still requires preformed adenine, methionine and histidine. This suggests that there is a further stage between *p*-aminobenzoic acid and adenine, methionine and histidine which 'Yeast 47' can, but *S. octosporus* cannot, negotiate.

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