Anaerobic growth, ergosterol content and sensitivity to a polyene antibiotic, of the yeast *Schizosaccharomyces japonicus*

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BULDER, C. J. E. A. 1971. Anaerobic growth, ergosterol content and sensitivity to a polyene antibiotic, of the yeast *Schizosaccharomyces japonicus*. Antonie van Leeuwenhoek **37**: 353–358.

Whereas *Saccharomyces cerevisiae* when grown in continuous culture under anaerobic conditions requires ergosterol, *Schizosaccharomyces japonicus* (syn. *Sch. versatilis*) can grow without this substance; sporulation is under anaerobic conditions hardly less prolific than under aerobic conditions.

The ergosterol content of anaerobically grown cells of *Sch. japonicus* was only 0.01 %, that of aerobically grown cells 0.24 %. Under anaerobic conditions, cell growth was hardly affected by the polyene antibiotic pimaricin; under aerobic conditions, *Sch. japonicus* is about as sensitive as *S. cerevisiae*. Qualitatively, the antagonistic effect of ergosterol on pimaricin action is the same in *Sch. japonicus* and in *S. cerevisiae*.

The relation between ergosterol content and the polyene sensitivity strongly confirms existing views (see Kinsky, 1967) on the mechanism of action of polyene antibiotics.

INTRODUCTION

Andreasen and Stier (1953, 1954) demonstrated that a supply of both ergosterol and oleic acid in the medium were indispensable for growth of *Saccharomyces cerevisiae* below a certain oxygen tension, in fact under very anaerobic conditions. Their results suggested that (1) molecular oxygen is needed for one or more steps in the biosynthesis of ergosterol and that (2) ergosterol is an essential component of the yeast cell. Indeed, the unravelling of the pathway of sterol biosynthesis showed that molecular oxygen is required for the oxidative cyclization of squalene to lanosterol and for the subsequent oxidative removal of three methyl groups in the transformation to cholesterol; ergosterol is believed to be synthesized mainly along the same pathway (see Popják and Cornforth, 1960).

Further, some insight into the function of ergosterol in yeasts and other fungi was gained by investigations into the mode of action of polyene antibiotics on fungi; it appeared that ergosterol, as a component of the cell membrane, was indispensable for maintaining the required permeability properties of that membrane (see Kinsky, 1967). It struck the present author that these recent additions to our knowledge of the biosynthesis and the function of ergosterol make *Schizosaccharomyces japonicus* (syn. *Sch. versatilis*) a very interesting yeast as it can grow under conditions too anaerobic to allow growth of *Saccharomyces cerevisiae*, and indeed, of all other yeasts tried (Bulder, 1963). The investigation to be reported here is concerned with characteristics of the anaerobic growth of *Schizosaccharomyces japonicus*, its content of ergosterol, and its sensitivity to the polyene antibiotic pimaricin (natamycin).

MATERIALS AND METHODS

Strains. Saccharomyces cerevisiae CBS 1172 (respiratory sufficient), *S. cerevisiae* "petite" (respiratory deficient) and *Schizosaccharomyces japonicus* var. *japonicus* CBS 354 (respiratory deficient species; Bulder, 1963, 1964).

Medium. Glucose 10 g, casamino acids 2.5 g, KH_2PO_4 1.2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, in 1 liter of distilled water; vitamins and compounds supplying trace elements as in Wickerham's basal media for carbon and nitrogen assimilation tests (Wickerham, 1951); pH 4.8.

Pimaricin. Pimafucin (registered trade mark Mycofarm-Delft) $2\frac{1}{2}\%$ suspension.

Analytical procedures. Dry weight was determined by centrifuging, washing, and drying at 100 C. Cell counts were made in a Thoma chamber. Ergosterol content was determined according to Hummel (Hoppe, 1960); the freeze-dried cell material was disrupted by boiling in a 5% solution of KOH in methanol.

Continuous culture arrangement for anaerobic growth: A block diagram is given in Fig. 1. The medium reservoir A is permanently flushed with a standard quality N_2 (less than 50 vpm[1] O_2) in order to keep dissolved oxygen at a low initial concentration. The medium is metered by a hose pressure pump B to the equilibrating vessel C through which high-purity N_2 is passed at a rate of 100 ml/min (less than 1 vpm O_2); the flows of gas and liquid from C to the continuously stirred fermentor D are combined to prevent upstream growth of cells; the fermentor is an erlenmeyer flask of 150 ml working volume, magnetically stirred. In a second erlenmeyer flask E, gas and liquid separate and the liquid is

[1]) vpm: volume per million volumes.

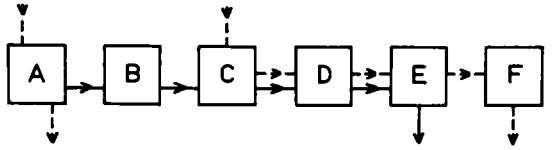

Fig. 1. Block diagram of anaerobic continuous culture arrangement. Dash-lines: gas flow; Solid lines: liquid flow. A: medium reservoir; B: pump; C: vessel for equilibrating oxygen pressure in gas and liquid; D: fermentor; E: drain; F: galvanic system for measuring oxygen partial pressure; further details in text.

eventually drained off. The gas is freed from CO_2 in a washing bottle by a 5% KOH solution when entering the oxygen measuring system F; this system consists of a Hersch-type galvanic cell provided with an electrolytic calibration cell and a gas-flow meter (for details see Hersch, 1956). With a high-purity N_2 supply (O_2 concentration when leaving the reducing valve on the cylinder measured 0.6–0.8 vpm), and with stainless-steel capillary or glass tubing with ground joints where possible, the oxygen concentration in the gas leaving the culture could be kept at ca. 2 vpm.

EXPERIMENTS AND RESULTS

Anaerobic growth. The ability of *Sch. japonicus* to grow under a low partial pressure of oxygen (1 to 3×10^{-6} at) was compared with that of a normal and a respiratory deficient strain of *S. cerevisiae*. In a continuous culture at a dilution rate of 0.1 hr^{-1} the two strains of *S. cerevisiae* could be kept permanently growing only when ergosterol and tween 80 were added to the medium but not without this addition. This confirms the results of Andreasen and Stier (1953, 1954). *Sch. japonicus*, however, could be grown without tween 80 and ergosterol; the cell density in steady state was 0.8 mg (dry) per ml, representing a yield on glucose of 8%, which is compatible with fermentation along the Embden Meyerhof pathway. Microscopical observation revealed several asci among the vegetative cells and the short hyphae of *Sch. japonicus*.

Ergosterol content. The apparent absence of any requirement for ergosterol in anaerobic growth of *Sch. japonicus* raised the question whether cells of this organism did contain any ergosterol. Ergosterol content was determined in cells grown anaerobically in batch culture, harvested by centrifugation and subsequently freeze-dried. The amount of ergosterol found, 0.01%, is extremely low if not essentially zero. However, in view of the sensitivity of aerobically grown *Sch. japonicus* towards pimaricin (see next paragraph), such cells were

also analyzed; these contained 0.26% ergosterol, a rather low but sensible amount.

Sensitivity to pimaricin. A polyene antibiotic, e.g. pimaricin, is supposed to exert its action on fungi by reacting with ergosterol present in the cell membrane; addition of ergosterol to the medium antagonizes the antibiotic action of the polyene (see e.g. Kinsky, 1967). The effect of different concentrations of pimaricin and added ergosterol on *Sch. japonicus* and *S. cerevisiae* (normal strain) was investigated in aerobic 50 ml batch cultures. The results are given in Tables 1 and 2. These tables show that pimaricin has an antibiotic action on *Sch. japonicus* as well as on *S. cerevisiae*, although *Sch. japonicus* appears to be somewhat less sensitive. In both yeasts, added ergosterol antagonizes the action of pimaricin. This result, suggesting the presence of ergosterol in *Sch. japonicus*, prompted the analysis of aerobically grown cells, the result of which has already been given in the preceding paragraph. Anaerobically grown cells of *Sch. japonicus* might be less sensitive to pimaricin than are aerobically grown cells. In order to investigate whether such a difference exists, this yeast was cultivated in two parallel continuous cultures at a dilution rate of 0.1 hr^{-1}, one culture aerobic and the

Table 1. Effect of pimaricin on growth of *Sch. japonicus* and antagonistic action of ergosterol on this effect.

Ergosterol added (μg/ml)	Number of days after which growth became visible		
	Pimaricin added		
	1 μg/ml	3 μg/ml	10 μg/ml
0	7	14	> 20
1	3	14	> 20
5	3	14	> 20
25	2	3	14

Table 2. Effect of pimaricin on growth of *S. cerevisiae* and antagonistic action of ergosterol on this effect.

Ergosterol added (μg/ml)	Number of days after which growth became visible		
	Pimaricin added		
	1 μg/ml	3 μg/ml	10 μg/ml
0	11	> 20	> 20
1	7	14	> 20
5	7	14	> 20
25	4	> 20	> 20

other anaerobic. After two days, when the cultures could be considered to have reached the steady state (cell concentration ca. 1.5×10^7/ml), 10 μg of pimaricin per ml were added to the medium reservoir of each culture. The cell density of the aerobic culture fell to less than 10^5/ml within the next three days, proving the practical absence of aerobic growth in the presence of pimaricin. The anaerobic culture showed an unchanging density, albeit the cells were somewhat malformed.

DISCUSSION

The results of the present study demonstrate that the viability of *Sch. japonicus* is not sensibly impaired when lack of oxygen results in a practical absence of ergosterol in the cell membrane, in contrast to other yeasts, e.g. *Saccharomyces cerevisiae*, that require an external source of ergosterol when a low oxygen tension prevents endogenous sterol synthesis. *Sch. japonicus*, if grown under aerobic conditions, is about as sensitive to pimaricin as are other fungi, but highly insensitive to this polyene antibiotic when, due to anaerobic growth conditions, ergosterol is absent in the cells. A comparable case of polyene sensitivity has been reported for *Mycoplasma laidlawii* (Weber and Kinsky, 1965; Feingold, 1965). The experiments with *M. laidlawii* and the presently reported experiments with *Sch. japonicus* differed in several respects; e.g. a pleuropneumonia-like organism versus a fungus; filipin, amphotericin and cholesterol in *M. laidlawii* versus pimaricin and ergosterol in *Sch. japonicus*; incorporation of sterol from the medium in *M. laidlawii* versus oxygen-dependent biosynthesis of sterol in *Sch. japonicus*; and the demonstration of sensitivity to the antibiotic by lysis of a washed culture versus inhibition of growth in a continuous culture. Nevertheless essentially they agree with the hypothesis (Weber and Kinsky, 1965) that the presence of sterol in the cell membrane is a prerequisite for polyene sensitivity; the very differences in experimental approach outlined before strengthen the evidence in favor of this hypothesis. It remains obscure to what extent the prolific sporulation – unusual in respiratory deficient yeasts (Ephrussi, Hottinguer and Tavlitzki, 1949) –, and the capacity for anaerobic sporulation of *Sch. japonicus* – unusual among the yeasts in general – have any relation to the facultative presence of ergosterol in this yeast – probably also an unusual property. It is difficult to accept that these features – possibly unique among the yeasts or even among the fungi in general – should meet in one species by mere coincidence. This species, moreover, is also intriguing because of cytological features already stressed by Wickerham and Duprat: "a remarkable fission yeast" (1945), and by Robinow: "the nucleus... is, so far, without counterpart among fungal nuclei" (1969).

The skilful technical assistance of Miss Marry Reinink is gratefully acknowledged.

Received 5 *February* 1971

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