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Effect of Clomiphene on the Content of Sterols and Fatty Acids in *Saccharomyces cerevisiae*

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ABSTRACT. During cultivation of *Saccharomyces cerevisiae* clomiphene regulates both quantitative and qualitative production of sterols and fatty acids as identified by gas chromatography and mass spectrometry. The content of sterols decreases to 75 %, the production of fatty acids is comparable with that in the control. The occurrence of sterols increases; sterols with methyl group in position 4, without double bond in position 22 and with double bond in position 24(25) or 24(28) predominate. Among fatty acids shorter saturated and monoene acids are primarily produced, 2-hydroxy acids practically disappeared.

The inhibition of lipid biosynthesis in fungi using sterols as models has been extensively studied (Rattray *et al.* 1975). Species that are easily cultivated and produce relatively high quantities of sterols, *e.g.* yeasts of the genera *Saccharomyces* or *Candida*, are used most frequently. The effect of fungicides — inhibitors of sterol biosynthesis — has also been investigated (Walsh and Sisler 1982). All hitherto known inhibitors block primarily the biosynthesis at the level of intermediates between lanosterol and ergosterol leading to the accumulation of reaction intermediates. This fact can be utilized *e.g.* for the biosynthesis of less common sterols and enhancement of fungicidal effects (growth is limited and the synthesis and composition of the cell wall are impaired).

In the present work, the regulation of the biosynthesis of lipids, in addition to proteins and polysaccharides one of the most important components of yeasts, was investigated; clomiphene (α-chloro-β-[4-(2-diethylaminoethoxy)-phenyl]stilbene), the effective compound of the antiestrogenic preparation Gravosan (*SPOFA*, Czechoslovakia) served as inhibitor.

MATERIAL AND METHODS

Saccharomyces cerevisiae CCY 21-4-63 was cultivated in a medium according to Olson and Johnson (1949), 2 % sucrose served as carbon source. The effect of clomiphene on growth was determined after a 1-d cultivation on a reciprocal shaker. Laboratory fermentor LF 2 (*Developmental Workshops, Czechoslovak Academy of Sciences*, Czechoslovakia) containing 2 L of the medium

TABLE I. Effect of clomiphene on growth of the culture, and content of sterols and fatty acids

Clomiphene	Flasks	Fermentor		
mg/L	biomass g/L	biomass g/L	sterols[c] %	fatty acids[c] %
0[a]	4.8	4.3	1.2	2.4
5	5.3	—	—	—
10	4.9	4.0[b]	0.9	2.5
16	1.3	—	—	—
32	1.1	—	—	—
64	1.1	—	—	—

[a] Control.
[b] Initial concentration 0.5 g/L.
[c] Related to biomass.

served for the production of biomass. Cultivation conditions: stirring frequency 30 Hz, temperature 30 °C, aeration 1.7 L/min, cultivation time 1 d.

Cells grown in the fermentor were centrifuged, washed twice with water, and hydrolyzed for 3 h with boiling 3 M KOH. The mixture was extracted three times with 300 mL of diethylether, acidified with HCl to pH 1 and extracted again with three 300-mL portions of diethylether. The extracts were dried and evaporated to dryness. Sterol acetates were prepared from the alkaline extract (by standing in a pyridine—acetanhydride solution for 1 d) and methyl esters were produced from the acidic extract (by reaction with BF_3—methanol with boiling for 1 min). Pyrrolidides were prepared according to Andersson (1978).

TABLE II. Effect of clomiphene on fatty acid content in *S. cerevisiae*

Acid[a]	Cultivation	
	with clomiphene	without clomiphene
12 : 0	2.3	1.1
14 : 0	4.1	2.7
15 : 0	1.3	0.5
15 : 1	2.1	0.2
16 : 0	4.9	5.8
9-16 : 1	64.2	55.4
18 : 0	1.8	3.2
9-18 : 1	17.2	27.9
9,12-18 : 2	tr	0.7
20 : 0	0.1	0.1
22 : 0	tr	0.4
24 : 0	tr	tr
26 : 0	tr	1.1
2-OH-20 : 0	tr	tr
2-OH-22 : 0	tr	tr
2-OH-24 : 0	tr	0.2
2-OH-26 : 0	tr	0.7

[a] Number before dash designates double bond position; 2-OH — 2-hydroxy acid.

TABLE III. Effect of clomiphene on composition of sterols in *S. cerevisiae* (%)

Sterol	Cultivation	
	with clomiphene	without clomiphene
Cholesta-8,24-dienol	6.0	4.6
4α-Methylcholest-8-enol	2.1	1.4
Cholesta-7,24-dienol	1.5	1.4
Ergosta-5,7,22-trienol	56.9	66.4
Ergosta-5,7,22,24(28)-tetraeno	12.2	21.4
Ergost-8(14)-enol	6.4	2.7
Ergostanol	0.9	—
Ergosta-8,24(28)-dienol	8.8	2.1
Ergosta-7,24 (28)-dienol	2.0	—
Unknown[a]	0.5	—
4α-Methylergost-7-enol	0.7	—
4α,14-Dimethylergosta-8,24-dienol	0.6	—
24-Methyllanost-8-enol	0.4	—
Not identified	1.0	—

[a] 4-Methylergostadienol with one bond in rings and other in side chain.

The mixtures were separated and identified in the apparatus GC—MS Hewlett-Packard 5995 B (CA, USA). Methyl esters were separated on a capillary fused silica column of 25 × 0.25 mm ID with a Carbowax phase (0.2 μm). Initial temperature was 150 °C, final temperature 220 °C, temperature increase 3 K/min. Pyrrolidides and sterol acetates were separated on a capillary fused silica column of 25 m × 0.25 mm ID with OV-101 (0.2 μm) or on the same column long 12.5 m. Temperature was programmed from 150 or 200 to 280 °C at a rate of 10 or 5 K/min. Ionization energy was 11 aJ (70 eV).

RESULTS

Table I summarizes data concerning the biomass production as related to the amount of added inhibitor and the composition of lipid fractions in the yeast cultivated with clomiphene and without it.

Fatty acids were identified and quantified by means of the method of GC—MS methyl esters (Řezanka *et al.* 1983); positions of double bonds in pyrrolidides were determined according to Andersson (1978). After the application of clomiphene the content of lower fatty acids (up to C_{16}) increased significantly, on the other hand, a remarkable decrease of C_{18} acids, linoleic acid in particular, was detected (Table II). However, disappearance of fatty acids above C_{20}, including 2-hydroxy acids, is the most interesting phenomenon.

The structure of sterols was determined on the basis of their retention times (Itoh *et al.* 1982) and interpretation of mass spectra (Knights 1967). During cultivations with clomiphene, 12 completely identified sterols were detected, one was identified only partially and 2 sterols were not identified at all, on the contrary, 7 completely identified sterols were detected in the control (Table III).

DISCUSSION

After the addition of clomiphene to the *Saccharomyces cerevisiae* culture it is possible to observe a change in the overall amount of sterols and quantitative and qualitative composition of sterols.

Table I illustrates the quantitative regulation of growth and production of individual components of the lipidic fraction. At low clomiphene concentrations the growth is rather stimulated than inhibited. Similar conclusions were also reached by Walsh and Sisler (1982) with many inhibitors — fungicides — tested with *Ustilago maydis*. This fact has not yet been clarified. Where scaling up, the biomass production decreases, however, in this case this phenomenon occurs even in the control. At variance with literature data the production of overall sterols decreases (Table I *vs.* Campagnoni *et al.* 1977), however, this finding is in agreement with similar experiments with the green alga *Chlorella kessleri* (Řezanka *et al.* 1985). The amount of fatty acids doesn't change, as also observed by us (Řezanka *et al.* 1985) with clomiphene and by Pierce *et al.* (1978) with nystatin. With respect to the detected decrease of sterol production and preserved fatty acid production it may be assumed that clomiphene inhibits a biosynthetic pathway branching off the oligoketide pathway.

Another type of regulation is manifested by a qualitative and quantitative shift in the occurrence of individual representatives of the studied components of the lipidic fraction. The regulatory effect of clomiphene on individual fatty acids content is illustrated by Table II. The increase of the content of fatty acids up to C_{16}, both saturated and monoenic, is most remarkable. On the contrary, the content of unsaturated C_{18} acids and, generally, acids with chain length of C_{18} and more sharply decreases; hydroxy acids practically disappear. Only Pierce *et al.* (1978) described a minor increase of the content of fatty acids after the inhibition by polyene antibiotics. Therefore, we assume that clomiphene (with respect to the way of biosynthesis of unsaturated and long fatty acids) inhibits both dehydrogenation of acids and their elongation above C_{18}.

The inhibition of splitting methyls in positions $C_{(4)}$ and $C_{(14)}$ is the main effect of clomiphene, together with the shift of double bond from position $C_{(8)}$ to position $C_{(7)}$ and formation of 5,7-diene conjugated system in sterols. These facts are in agreement with literature data (Campagnoni *et al.* 1977; Hosokawa *et al.* 1984). On the contrary, the introduction of double bond to position 8(14) and reduction to a fully saturated skeleton are stimulated. Alkylation on $C_{(24)}$ and reduction of double bond 24(28) are not influenced. Irrespective of the similarity of the chemical structure of triparanol and clomiphene different biochemical pathways leading to sterols were apparently influenced. The addition of clomiphene resulted in a pronounced increase of the content of sterols at the initial steps of the biosynthetic pathway leading to ergosterol. The fact that clomiphene as inhibitor is not strictly specific has already been demonstrated (Řezanka *et al.* 1985).

It may be discussed, whether the effect of clomiphene on the content of fatty acids is not rather a secondary manifestation of the changed structure of cell walls caused by the presence of other sterols than by interference of clomiphene with the oligoketide pathway during fatty acid synthesis. However, this assumption will have to be further verified.

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