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OPTIMIZATION OF ERGOSTEROL BIOSYNTHESIS BY *SACCHAROMYCES CEREVISIAE* – CHOICE OF OBJECTIVE FUNCTION

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Key words: ergosterol, $\Delta^{5,7}$ - sterols, *Saccharomyces cerevisiae*, continuous culture, fed-batch culture, optimization criterion

The objective of the study was to optimize ergosterol production by *Saccharomyces cerevisiae* in continuous and fed-batch cultures. Use was made of three optimization criteria: ergosterol yield related to the mass unit of the substrate supplied to the fermenter, and two criteria proposed for the purpose of the study, both incorporating (in addition to ergosterol yield) ergosterol content in yeast cells and proportion of ergosterol in the total $\Delta^{5,7}$-sterols content.

In both culture types, specific growth rate was found to affect the content of $\Delta^{5,7}$-sterols in the yeast cells. In continuous culture, three of them (ergosterol, dehydroergosterol, and unidentified sterol) reached their maximum at the dilution rate of 0.74, 0.102 and 0.131 h^{-1}, respectively. For ergosterol and for the unidentified sterol, these were the dilution rates at which ethanol content in the culture medium began to increase. Dihydroergosterol content was an increasing function of dilution rate. In the fed-batch culture with purely oxidative assimilation of glucose, $\Delta^{5,7}$-sterols content in yeast cells (except that of dehydroergosterol) increased with increasing specific growth rate.

Optimization carried out with the three objective functions mentioned above showed that they reached their maxima at essentially the same argument values – both in continuous and fed-batch cultures. This indicates that the ergosterol yield criterion can be substituted for the two, more sophisticated, optimization criteria. The optimum dilution rate for continuous culture was 0.13 h^{-1}, and the optimum time of fed-batch culture ranged between 6 and 8 h.

INTRODUCTION

Ergosterol, which has been identified as the major $\Delta^{5,7}$-sterol synthesized by the cells of *Saccharomyces cerevisiae*, is an interesting raw material for the production of vitamin D_2 [Arnezeder & Hampel, 1990]. However, in the course of ergosterol biosynthesis, other $\Delta^{5,7}$-sterols (dehydroergosterol, dihydroergosterol and one unidentified sterol) pass from the yeast cells into the extract, thus producing contamination. The amount of ergosterol, as well as that of the other $\Delta^{5,7}$-sterols in the cells of *Saccharomyces cerevisiae*, varies in a comparatively wide range and is affected by a variety of factors, *e.g.* by growth rate [Arnezeder & Hampel, 1990; Behalova *et al.*, 1994; Hunter & Rose, 1972], by the type of the carbon source in the medium [Novotny *et al.*, 1988], by the carbon-to-nitrogen ratio [Arnezeder & Hampel, 1990; Novotny *et al.*, 1987] and by the ethanol content in the culture medium [Herve *et al.*, 1994; Sajbidor *et al.*, 1995; Vedlichova *et al.*, 1998; Walker-Caprioglio *et al.*, 1990].

Because of the variable content of $\Delta^{5,7}$-sterols in the yeast cells, the yield criterion – defined as the ergosterol quantity obtained per mass unit of substrate supplied to the fermenter – may become inadequate when assessing the efficiency of ergosterol production by yeasts. Optimizing the process in terms of the yield criterion (objective function), it cannot be excluded that the maximum ergosterol yield will be achieved when biomass harvest is high at a low ergosterol content, or when the accumulation of the other $\Delta^{5,7}$-sterols in the yeast cells is high. In both instances, the biosynthesis of ergosterol by yeast cells would involve high costs.

The objective of the present study was to optimize the process of ergosterol biosynthesis by *Saccharomyces cerevisiae* in continuous and fed-batch cultures, making use of ergosterol yield as the optimization criterion, as well as of some other objective functions incorporating the purity of the extracted ergosterol and the content of ergosterol in the yeast cells.

MATERIALS AND METHODS

YEASTS: Use was made of the *S. cerevisiae* D7 strain from the collection of the Department of Fermentation Chemistry and Bioengineering, Institute of Chemical Technology, Prague, Czech Republic. The strain was stored on agar slants at 4°C.

CULTURE MEDIUM: The composition of the medium was established in a previous study [Garncarek *et al.*, 1997]. In the fed-batch culture, the medium consisted of (g/L) glucose, 250; $(NH_4)_2SO_4$, 15; yeast extract, 62.5; $MgSO_4 \cdot H_2O$,

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6.25, and $CaCl_2$, 1.25. In the continuous culture, the medium was diluted 12.5 fold. The proportion of the components was the same as in the fed-batch culture.

GROWTH CONDITIONS: Both continuous and fed-batch cultures were carried out at 30°C, pH 4.8 and agitation speed of 600 rpm. For pH adjustment use was made of 5% NaOH solution, which was dosed automatically into the fermenter. Continuous cultures were done in a 1.8 L bioreactor made by BIOTEC. Fed-batch cultures were carried out in a fermenter designed in the Wrocław University of Economics with an initial working volume of 5 L.

ANALYTICAL METHODS: Biomass concentration was measured turbidimetrically at 735 nm wavelength, making use of the calibration curve plotted for the *S. cerevisiae* D7 strain. Glucose concentration and total nitrogen in the culture medium was determined enzymatically via a Comray set and by the Kjeldahl's method, respectively. Merck test stripes were used to determine ammonia nitrogen. Nitrogen and carbon content in the yeast extract was established with a CHN analyzer of EA 1108 type. Ethanol concentration in the culture medium was measured by gas chromatography, using a flame ionization detector. Sterol content in the yeast cells was determined by HPLC, after alkaline hydrolysis and sterols extraction. The equipment consisted of a C18 column and a UV detector (282 nm), the mobile phase being a methanol–water-ethanol (86:10:4) system at room temperature. The velocity of mobile phase flow equalled 2 mL/min.

RESULTS AND DISCUSSION

CONTINUOUS CULTURES

Continuous cultures were carried out at a wide range of dilution rate (D), which varied from 0.03 to 0.233 h^{-1}. Each of them was carried out over a period, which was at least five times as long as the residence time (inverse of dilution rate), and biomass concentration was controlled in transient state. Under steady state conditions, the following parameters were determined – biomass concentration, glucose content, total nitrogen and ethanol in the culture medium, as well as the levels of particular $\Delta^{5,7}$-sterols in the yeast cells.

Table 1 shows the effect of the dilution rate on biomass concentration, as well as on glucose content, total nitrogen and ethanol concentration in the culture medium. The plots of Fig. 1 and Fig. 2 illustrate the response of ergosterol and dehydroergosterol and the response of dihydroergosterol and an unidentified sterol in yeast cells to the increase in dilution rate, respectively.

TABLE 1. Effect of dilution rate on biomass concentration, as well as glucose content, total nitrogen and ethanol content in the medium of the continuous *S. cerevisiae* culture.

No of culture	D [h^{-1}]	Biomass concentration [g/L]	Glucose content [g/L]	Total nitrogen content [mg/L]	Ethanol content [mg/L]
1	0.030	8.66	12.5	123.5	0.197
2	0.053	10.12	18.0	109.1	0.116
3	0.074	10.12	23.0	116.9	0.173
4	0.102	10.03	16.4	120.0	0.951
5	0.131	10.15	16.4	123.7	1.851
6	0.152	9.00	15.1	126.0	2.147
7	0.156	8.35	17.5	119.7	2.048
8	0.179	8.23	16.5	127.3	2.463
9	0.233	7.94	36.1	131.3	2.925

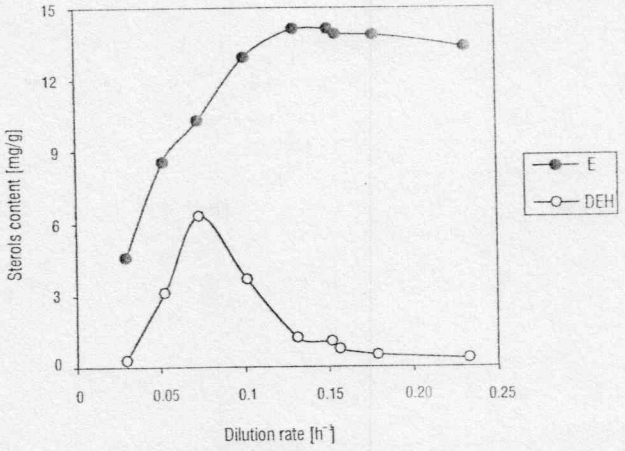

FIGURE 1. Effect of dilution rate on ergosterol and dehydroergosterol content in yeast cells; E=ergosterol, DEH=dehydroergosterol.

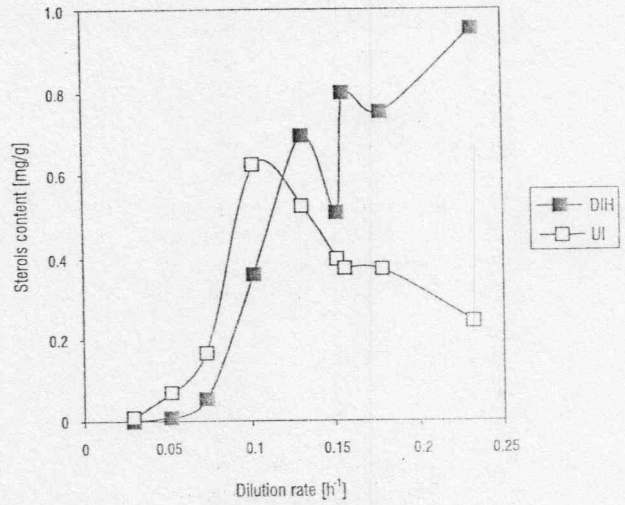

FIGURE 2. Effect of dilution rate on the content of dihydroergosterol and unidentified sterol in yeast cells; DIH=dihydroergosterol, UI=unidentified sterol.

As shown by the data of Table 1, the depletion of nitrogen was incomplete, and the composition of the medium was indicative of growth limitation by the nitrogen source (the molar ratio of C to N amounting to 13.8). Ammonia nitrogen content in the culture medium was below 20 mg/L, which indicates that not all of the nitrogen components of the yeast extract are available.

The shapes of the curves in Figs. 1 and 2 substantiate a distinct influence of the specific growth rate on the synthesis of the investigated sterols. The results obtained for ergosterol are similar to those achieved by Arnezeder and Hampel [1990] with a nitrogen-limited culture (the C/N ratio amounted to 30). This means that in both the studies the accumulation of ergosterol in the yeast cells followed a similar pattern: ergosterol content increased with increasing

dilution rate, and began to decrease slowly once the maximum value had been reached. However, the response of ergosterol content to the increase in the dilution rate differed totally when the culture was carbon-limited [Arnezeder & Hampel, 1990; Hunter & Rose, 1972] and the C/N ratio in the glucose medium amounted to 2.2, as well as when cultivation was carried out with a modified Olson-Johnson [1949] medium [Behalova *et al.*, 1994]; in both the cases ergosterol content decreased with increasing dilution rate.

As it may be inferred from the plots of Figs. 1 and 2, the content of dehydroergosterol and that of the unidentified sterol also reach their maximal values at certain specific growth rates, and these maxima are far more distinct than the maximum of ergosterol content. The plot of dihydroergosterol response is increasing function of dilution rate. Behalova *et al.* [1994] reported different results – in their study the content of both dehydroergosterol and unidentified sterol decreased with rising specific growth rate whereas dihydroergosterol content reached a maximum after a certain value of the specific growth rate had been achieved. The results obtained by Behalova *et al.* [1994] are not comparable with those plotted in Figs.1 and 2. Their investigations involved a narrow range of dilution rate, which varied from 0.05 to 0.125 h^{-1}. Furthermore, on the basis of the information provided in their paper it is very difficult, if at all, to define the relationship between carbon content and nitrogen content in the culture medium. It should, however, be noted that, apart from the study by Behalova *et al.* [1994], the available literature does not contain references to the synthesis (by any yeast strain cultivated continuously) of individual $\Delta^{5,7}$-sterols which would parallel ergosterol during extraction from the yeast cells.

FED-BATCH CULTURES

Fed-batch cultures were carried out for 10 or 12 h by making use of an identical, a priori established profile of medium dosage. It was anticipated that medium supply to the yeast should follow the sigmoid curve, because biomass growth in a fed-batch culture is described by the sigmoid curve. Thus, the time (t)-dependence of the total volume (V_T) of the medium supplied to the fermenter takes the form

$$V_T(t)=a+b/[1+\exp(-(t-c)/d)] \quad (1)$$

where a,b,c and d are parameters.

The values of a,b,c and d were determined by nonlinear regression, making use of the data obtained in an earlier study [Miśkiewicz & Wilczyński, 1996] with a culture of another *S. cerevisiae* strain, which was controlled by the level of dissolved oxygen.

In the fed-batch cultures, determinations were carried out every 2 h for the same parameters as in the continuous cultures. Table 2 includes the results obtained with a 10-hour fed-batch culture, *i.e.* it shows the time-dependent response of biomass concentration, glucose content, total nitrogen and ethanol content. Figures 3 and 4 show the plots describing the cellular content of the investigated $\Delta^{5,7}$-sterols, as well as the plot of the specific growth rate calculated using the procedure specified below: The experimental values obtained for the biomass were fitted with the sigmoid curve in terms of nonlinear regression, thus yielding a function which relates biomass concentration to time, X(t). Then, the specific growth rate (μ) was calculated analytically by virtue of

$$\mu=(dX/dt)/X \quad (2)$$

TABLE 2. Time-dependent response of biomass growth, glucose content, total nitrogen and ethanol content during fed-batch culture of *S. cerevisiae*.

Duration of culture [h]	Biomass concentration [g/L]	Glucose content [mg/L]	Total nitrogen content [mg/L]	Ethanol content [mg/L]
0	2.63	0	70.0	0
2	3.29	25.5	76.0	66.3
4	4.24	50.0	82.4	63.9
6	5.70	55.7	140.1	54.1
8	8.08	8.1	135.5	63.9
10	9.95	25.9	116.7	157.2

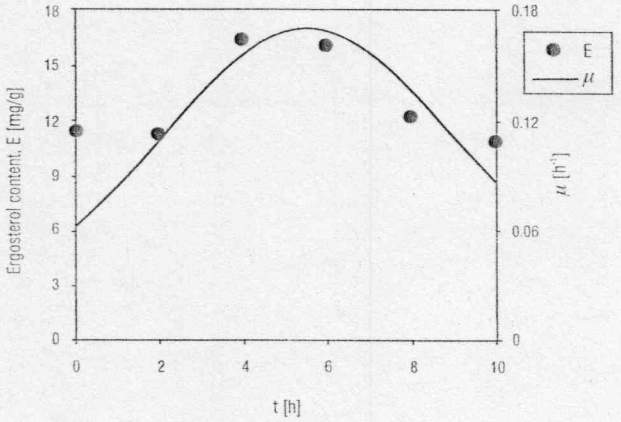

FIGURE 3. Accumulation of ergosterol in yeast cells during fed-batch culture.

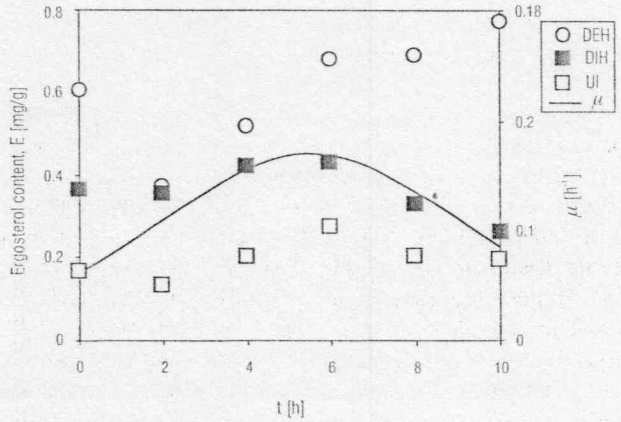

FIGURE 4. Accumulation of dehydroergosterol, dihydroergosterol and unidentified sterol in yeast cells during fed-batch culture; DEH=dehydroergosterol, DIH=dihydroergosterol, UI=unidentified sterol.

The data of Table 2 demonstrate that glucose has been assimilated almost completely, like in the continuous culture. The quantity of nonassimilated total nitrogen is also similar to the one measured in the continuous cultivation procedure. The poor ethanol content in the culture medium evidences an oxidative metabolism of glucose by the yeasts.

As shown by the plots of Fig. 3 and 4, there is also a correlation between specific growth rate and cellular $\Delta^{5,7}$-sterols content in the fed-batch culture. This correlation holds particularly for dihydroergosterol, ergosterol and the unidentified sterol; the rise in specific growth rate increases the degree of cellular accumulation. For dihydroergosterol, the said relationship follows the same pattern as in the continuous culture. For ergosterol and for the unidentified sterol, however, this pattern differs slightly as compared to that in the continuous culture where at certain dilution rates ($> 0.102\ h^{-1}$ and $> 0.13\ h^{-1}$ for the unidentified sterol and ergosterol, respectively) their accumulation in the yeast cells became inhibited. This difference might be attributed to the fact that at the above mentioned dilution rates, oxidative assimilation of glucose in the continuous cultures paralleled the fermentation process. According to some investigators [Pichova *et al.*, 1985], such conditions have no favourable effect on the synthesis of ergosterol. The available literature includes no references to the synthesis of the unidentified sterol under such conditions.

Other authors [Walker-Caprioglio *et al.*, 1990; Koukkou *et al.*, 1993] believe that the presence of ethanol in the culture medium may be regarded as the sole contributor to the decrease of ergosterol content in the yeast cells when cultivation is carried out under aerobic conditions. However, a contribution like that was observed only at ethanol concentrations over a dozen times as high as those involved in the continuous cultures described in the present paper.

OBJECTIVE FUNCTIONS

From the experimental data it can be inferred that, firstly the constant values of such cultivation parameters as temperature, pH, aeration conditions, and composition of feeding substrate do not guarantee a constant content of the investigated $\Delta^{5,7}$-sterols in the yeast cells and, secondly, that under such conditions cellular accumulation is influenced by the specific growth rate and by the physiological state of the yeast. The same factors affect the concentration of the biomass in the culture medium. Thus, it follows that the yield of ergosterol synthesis (assessed as the quantity of ergosterol produced by a mass unit of substrate supplied to the fermenter) depends on the dilution rate in the continuous culture and on the duration of the process in the fed-batch culture. The relation can be written as

$$Y_{E/S_0}(D)=E(D)*X(D)/S_0 \qquad (3)$$

and

$$Y_{E/S_0}(t)=[E(t)*X(t)*V(t)-E(0)*X(0)*V(0)]/[S_0*V_T(t)] \qquad (4)$$

for continuous culture and fed-batch culture, respectively, where: $Y_{E/S0}$ = ergosterol yield, E = ergosterol content in yeast cells (expressed in dimensionless units), X = biomass concentration, S_0 = substrate (glucose) concentration in the feeding medium, V = liquid volume in the fermenter, V_T = total volume of medium supplied to the fermenter, D = dilution rate, t = time.

A major objective in the synthesis of ergosterol from yeast is to maximize not only the ergosterol yield as related to the substrate fed to the fermenter, but also the ergosterol content in the yeast cells, as well as the proportion of ergosterol in the total content of $\Delta^{5,7}$-sterols. In general, however, these optimization criteria do not provide maximal values for the same argument. With these thoughts in mind, two synthetic objective functions (of a compromise nature) were proposed for assessing the efficiency of ergosterol production by the yeasts. One of these functions, Y_1, is the product of the three optimization criteria mentioned above and takes the form of

$$Y_1(D)=Y_{E/S_0}(D)*E^2(D)/[E(D)+DEH(D)+DIH(D)+UI(D)] \qquad (5)$$

and

$$Y_1(t)=Y_{E/S_0}(t)*E^2(t)/[E(t)+DEH(t)+DIH(t)+UI(t)] \qquad (6)$$

for continuous culture and fed-batch culture, respectively, where: DEH = dehydroergosterol content in yeast cells, DIH = dihydroergosterol content in yeast cells, UI = content of unidentified sterol in yeast cells; the notation of the other terms as in the relations of (3) and (4).The other objective function, Y_2, has been defined as the sum of the relative values of the following quantities: ergosterol yield, cellular content of ergosterol, and proportion of ergosterol in the total $\Delta^{5,7}$-sterols content. The relative values of interest have been obtained via dividing them by their maximal values achieved in the cultures. In this way, each of the three components is of the same rank in the objective function Y_2. Thus, we can write for the continuous culture

$$Y_2(D)=[Y_{E/S_0}(D)/Y_{E/S_0max}]+[E(D)/E_{max}]+[P(D)/P_{max}] \qquad (7)$$

For the fed-batch culture, the formal form of the objective function Y_2 incorporates only a different argument. Hence,

$$Y_2(t)=[Y_{E/S_0}(t)/Y_{E/S_0max}]+[E(t)/E_{max}]+[P(t)/P_{max}] \qquad (8)$$

where:

$$P(D)=E(D)/[E(D)+DEH(D)+DIH(D)+UI(D)],$$
$$P(t)=E(t)/[E(t)+DEH(t)+DIH(t)+UI(t)],$$

Y_{E/S_0max}, E_{max}, P_{max} = maximal values of Y_{E/S_0}, E and P, respectively; the notation of the other terms as in the relations (3), (4), (5) and (6).

Obviously, the objective of optimization for the two proposed criteria is their maximization.

The plots of Figs. 5 to 7 show the optimization of ergosterol synthesis with the use of all the objective functions. Figure 5 provides relevant data for the continuous culture, whereas Fig. 6 and Fig. 7 contains relevant results for a 10-hour fed-batch culture and 12-hour fed-batch culture, respectively, which differed in the amount of inoculum and in the content of cellular sterols at the moment of inoculation.

The results plotted in Figs. 5 to 7 are surprising in that the three objective functions reached their maxima at approximately the same argument value, irrespective of whether it was a continuous or a fed-batch culture. This finding indicates that ergosterol yield related to the mass unit of the feeding substrate is a universal optimization criterion in the biosynthesis of ergosterol by *S. cerevisiae*. And the finding also evidences that this criterion can be substituted for the objective functions which, besides ergosterol yield incorporate the cellular content of ergosterol and the proportion of ergosterol in the total $\Delta^{5,7}$-sterols content.

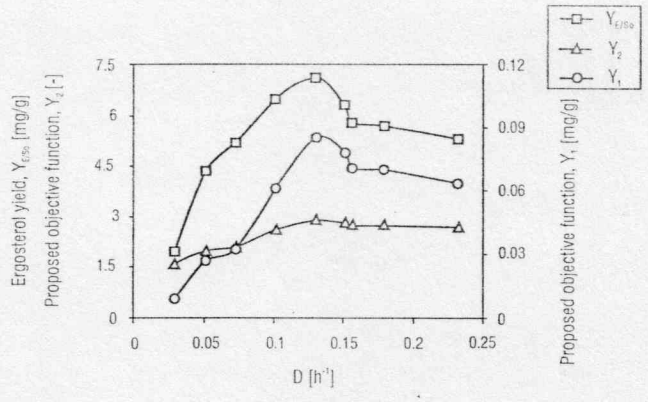

FIGURE 5. Optimization of ergosterol biosynthesis in continuous culture.

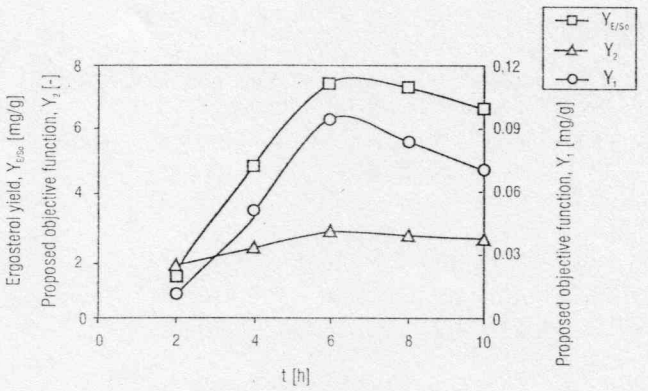

FIGURE 6. Optimization of ergosterol biosynthesis in 10-hour fed-batch-culture.

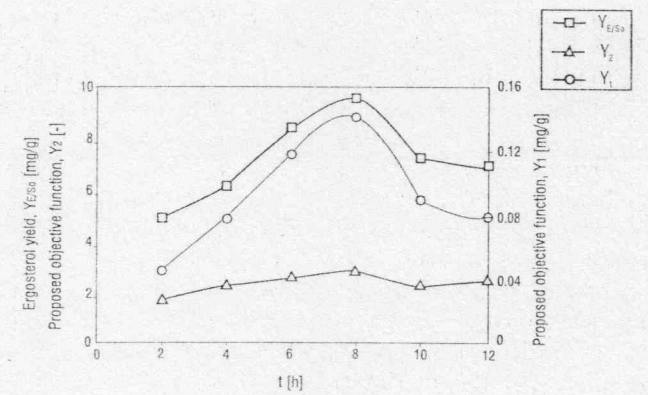

FIGURE 7. Optimization of ergosterol biosynthesis in 12-hour fed-batch-culture.

CONCLUSIONS

1. In a nitrogen-limited continuous culture of *Saccharomyces cerevisiae*, the content of $\Delta^{5,7}$-sterols (ergosterol, dehydroergosterol, dihydroergosterol and unidentified sterol) in the yeast cells is noticeably affected by the dilution rate. The content of ergosterol, dehydroergosterol and unidentified sterol reaches its maximum value at the dilution rate of 0.74, 0.102 and 0.131 h^{-1}, respectively, whereas dihydroergosterol content is an increasing function of dilution rate.
2. Ergosterol content and unidentified sterol content in *Saccharomyces cerevisiae* cells begin to decrease once the dilution rate has reached the value at which the concentration of ethanol in the culture medium begins to rise.
3. In fed-batch cultures with oxidative assimilation only, the content of sterols, except that of dehydroergosterol, increases with in the specific growth rate.
4. Ergosterol yield, defined as the quantity of ergosterol obtained per mass unit of the substrate supplied to the fermenter, is a universal objective function. It reaches its maximal values approximately at the same argument values as do the other two optimization criteria (proposed in this paper), which incorporate not only ergosterol yield, but also cellular ergosterol content and the proportion of ergosterol in the total content of $\Delta^{5,7}$-sterols in *Saccharomyces cerevisiae*.
5. Ergosterol yield was the highest at the dilution rate of 0.13 h^{-1} in the continuous culture, and between the 6th and 8th hour of the process in the fed-batch cultivation of *Saccharomyces cerevisiae* with glucose as a carbon source.

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OPTYMALIZACJA BIOSYNTEZY ERGOSTEROLU PRZEZ DROŻDŻE *SACCHAROMYCES CEREVISIAE* – DOBÓR FUNKCJI CELU

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Celem pracy była optymalizacja produkcji ergosterolu przez drożdże *Saccharomyces cerevisiae* zarówno w hodowli ciągłej jak i okresowej z dolewem porcjowym. Jako funkcje celu stosowano wydajność ergosterolu względem substratu doprowadzonego do fermentora oraz dwa inne, zaproponowane kryteria optymalizacyjne, które, oprócz wydajności ergosterolu, uwzględniają także jego zawartość w komórkach drożdży oraz jego udział w łącznej zawartości $\Delta^{5,7}$- steroli.

Na podstawie przeprowadzonych badań stwierdzono, że w obydwu typach hodowli występuje wyraźny wpływ właściwej szybkości wzrostu na zawartość poszczególnych $\Delta^{5,7}$- steroli w komórkach drożdży. W hodowli ciągłej zawartości trzech z tych steroli, ergosterolu, dehydroergosterolu oraz sterolu nieznanego, osiągnęły maksimum dla szybkości rozcieńczania wynoszących odpowiednio 0,74, 0,102 i 0,131 h^{-1}. W przypadku ergosterolu i sterolu nieznanego były to szybkości, powyżej których w środowisku hodowlanym wyraźnie rosła zawartość etanolu. Zawartość czwartego z $\Delta^{5,7}$- steroli, tj. dihydroergosterolu, była rosnącą funkcją szybkości rozcieńczania.

W hodowli okresowej z dolewem porcjowym, w której miało miejsce czysto tlenowe przyswajanie glukozy, zawartość poszczególnych $\Delta^{5,7}$- steroli w komórkach drożdży, oprócz dehydroergosterolu, zwiększała się wraz ze wzrostem właściwej szybkości wzrostu.

Dokonując optymalizacji przy pomocy trzech wspomnianych wcześniej funkcji celu okazało się, że osiągają one swoje maksima dla zbliżonych wartości argumentu. Dotyczy to zarówno hodowli ciągłych jak i okresowych z dolewem porcjowym. Można zatem wydajnością ergosterolu zastąpić kryteria bardziej skomplikowane. Optymalna szybkość rozcieńczania dla hodowli ciągłej wynosiła 0,13 h^{-1}, natomiast optymalny czas trwania hodowli okresowej z dolewem porcjowym wynosił od 6 do 8 godzin.