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Original paper

Modelling of ergosterol production by S. cerevisiae in presence of n-dodecane as oxygen-vector

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Abstract The previous studies on ergosterol production by *Saccharomyces cerevisiae* in presence of n-dodecane as oxygen-vector have been continued by mathematical modelling the fermentation process. In this purpose, the most efficient fermentation regime has been considered, namely fed-batch fermentation, and was based on the influences of hydrocarbon volumetric fraction, biomass concentration, and aeration rate on the ergosterol content inside the yeast cells. The model describing the fermentation process has been established by means of the statistical analysis, using a factorial experiment of second order. The considered variables control the ergosterol production in a 94.4% extent, the biomass concentration exhibiting the most important influence.

Keywords Ergosterol, *S. cerevisiae*, n-dodecane, oxygen-vector, air superficial velocity.

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Introduction

Ergosterol (ergosta-5,7,22-trien-3β-ol) is the precursor of vitamin D2 (ergocalciferol), because it possesses the ability to be converted under UV radiation to this vitamin (also named provitamin D2) (RAJAKUMAR, 2007). The functions of ergosterol in microbial or plant cells are similar to those of cholesterol in mammalian cells, namely the maintenance of the cellular membrane mechanical integrity, vital functions, and the normal activity of plasma-membrane proteins activity (BARD, 1993; PARKS, 1995). Among the multiple medical applications of ergosterol, its antitumor and antifungal activity can be underlined (ROBERTS, 2003).

The natural sources of ergosterol are mainly the yeasts (*Saccharomyces spp.*, *Candida spp.*), but it can also be found in fungus (*Claviceps spp.*) or plants (orchids) (BARD, 1993; PARKS, 1995; HUANG, 2011). In the main source, yeast cells, this compound is accumulated in membranes, mainly in the plasma-membrane as free ergosterol, or in membrane lipids as esters with fatty acids (VEEN, 2003; Shobayashi, 2005). The yeasts metabolic pathway for ergosterol biosynthesis is complex and involves multiple steps using specific enzymes dedicated to the conversion of squalene into this sterol (DAUM, 1998; SOUZA, 2011).

The chemical synthesis of ergosterol does not represent an efficient method for its production, due both to the low yield and to the multiple steps with high consumption of materials and energy (WU, 2012). Therefore, the most attractive alternative for producing ergosterol at larger-scale is the aerobic fermentation using *S. cerevisiae*, the substrate being especially glucose, but by-products from agriculture or food industry (WU, 2012; SHANG, 2006; BLAGA, 2018).

The previous studies on ergosterol biosynthesis concluded that the oxygen supply to the broth is the most important parameter that has to be considered in fermentation operating regime. The major role of oxygen is suggested by the mechanism of ergosterol biosynthesis proposed by Rosenfeld and Beauvoir (2003), which indicates that twelve molecules of oxygen are used in non-respiratory metabolic pathway to convert squalene into ergosterol in *S. cerevisiae* cells (ROSENFELD, 2003). In most cases, the bioreactor capacity to generate high rate of oxygen diffusion from air to the broths, or to ensure high level of dissolved oxygen, is limited due to its design and operational characteristics (LUPASTEANU, 2007).

However, for the biosynthesis of single-cell protein on various water insoluble hydrocarbon substrates, using conventional bioreactors, it was observed that the presence of an organic phase immiscible with water improves significantly the oxygenation efficiency, without needing supplementary energy consumption for stirring (CASCAVAL, 2006). These organic liquids were defined as oxygen-vectors and possess high ability to dissolve oxygen compared to aqueous media (the oxygen solubility in these compounds is from several to over thirty times higher than in aqueous broths). The main categories of oxygen-vectors tested in fermentations were hydrocarbons, perfluorocarbons, and oils (CASCAVAL, 2006; DUMONT, 2006; DA SILVA, 2008; LI, 2012).

Our previous studies on ergosterol production by *S. cerevisiae* in batch and fed-batch fermentation systems indicated that the addition of n-dodecane led to an increase of its final concentration for 1.5 times, the best results regarding the productivity being recorded for the fed-batch process (BLAGA, 2018).

These previous experimental investigations are continued and developed by modelling the most efficient fermentation systems for ergosterol production, namely fed-batch fermentation, by including the cumulated influences of biomass concentration, air superficial velocity, and n-dodecane concentration on ergosterol accumulation inside *S. cerevisiae* cells. The effects of the studied factors are quantitatively described using a mathematical model proposed by means of the statistical analysis.

Materials and Method

The experiments were carried out using a stirred bioreactor (Fermac, Electrolab), with 2 L total volume, provided with computer-controlled and recorded parameters. The bioreactor mixing system consists of one turbine impeller and three baffles. The bioreactor and impeller characteristics have been presented in the previous paper (BLAGA, 2018).

The sparging system consists of a perforated tube with 7 mm diameter, placed at 15 mm from the vessel bottom, having 4 holes with 1 mm diameter. The air volumetric flow rate was varied between 2 and 10 L/h (corresponding to an air superficial velocity of 5-25 x 10^{-5} m/s). The impeller rotation speed was maintained at 200 rpm. The dissolved oxygen concentration has been calculated as percent from the saturation level, according to the oxygen probe calibration.

According to the previous studies on the efficiency of ergosterol biosynthesis, the fermentation was carried out in fed-batch regime. In the experiments *S. cerevisiae* has been used. In order to obtain the inoculum, a plate culture (plate media: 20 g/L peptone, 20 g/L glucose, 10 g/L yeast extract, 12 g/L agar) of yeast cells has been grown at 30±1°C for 20 h (Vovsik, 2013). Then, the yeast cells were transferred into a 250 mL flask containing 50 mL of sterile culture medium and incubated for 20 h at 30±1°C and 180 rpm.

The stirred bioreactor contained 1 L working volume of a proper medium consisting of 60 g/L glucose, 31.2 g/L yeast extract, 7.8 g/L ammonium sulphate, 3.7 g/L potassium dihydrogen phosphate, 3.1 g/L magnesium sulphate, 1.25 g/L calcium chloride, 0.4 g silicon oil in tap water. After sterilization at 121°C for 20 min, the medium was inoculated with 5% vol. of inoculum. For the fed-batch

fermentation, 60 mL of 600 g/L glucose solution was added into the bioreactor every 30 min, in the purpose to maintain the glucose level at minimum 10 g/L (BLAGA, 2018). The temperature was maintained at 30ºC during the fermentation cycle. The pH-value was maintained at 5.4, being automatically adjusted by addition of 25% ammonia solution.

N-Dodecane (SIGMA Chemie GmbH) was used as oxygen-vector (density 750 g/L at 20°C, oxygen solubility $54.9 \cdot 10^{-3}$ g/L at 35ºC and atmospheric air pressure). The sterilized hydrocarbon was added into the bioreactor at the beginning of fermentation, its volumetric concentration into the broth varied between 0 and 15%.

For ergosterol extraction, 0.2 g dry cells have been treated with 10 mL alcoholic solution of potassium hydroxide obtained by solving 8 g potassium hydroxide into 32 mL 60% vol. alcoholic solution (WU, 2012). The extraction duration was 3 h at 80°C. After the extract was cooled at the room temperature, 10 mL of petroleum ether were added, the mixture being stirred for 2 min with a vortex. The phases were separated and 2 mL extract was subjected to evaporation. The extracted ergosterol was quantified by HPLC method (Dionex Ultimate 3000 system using a Lichrospher Si 100 column 250 x 4.6 mm, 5 μm), the mobile phase consisting of a mixture of n-hexane and tetrahydrofuran with volumetric ratio 85:15 and flow rate 1.0 mL/min (SHANG, 2006). The HPLC system was provided with PDA detector at 280 nm. The ergosterol content has been considered as percent from the biomass amount.

Each experiment has been carried out for at least three times, considering identical conditions, the average value of measured parameters being used. The maximum experimental error varied between of 4.83 and 5.55%.

Results and Discussion

According to the previous results, the biomass concentration was significantly increased in the fed-batch system compared to the batch fermentation, regardless of the presence and concentration of n-dodecane (Blaga, 2018). This difference is the consequence of the extension of period corresponding to the biomass exponential growth. Although Figure 1 indicates clearly the direct dependence between yeast cells concentration and ergosterol accumulation, the ergosterol content inside the *S. cerevisiae* cells is controlled also by n-dodecane volumetric fraction and, implicitly, dissolved oxygen concentration.

As it can be observed from Figure 1, the biomass amount accumulated over 20 hours of fermentation in presence of 15% vol. n-dodecane is lower than that recorded for 5 or 10% vol. hydrocarbon, this evolution being explained by the inhibitory effect induced at higher oxygen concentration. Similar to biomass concentration dependence on n-dodecane volumetric fraction, the maximum ergosterol concentration corresponds to 10% vol. n-dodecane.

The effect of aeration rate differs from ergosterol production to biomass accumulation. According to Figure 2, the biomass amount is continuously increased by increasing the air superficial velocity, due to the positive influence of dissolved oxygen on biomass growth rate. At hydrocarbon concentration superior to 5% vol., the influence of aeration intensification is diminished over 17×10^{-5} m/s, as the result of the oxygen-vector presence which allows to increasing the level of dissolved oxygen concentration inside the broth even at lower aeration rate.

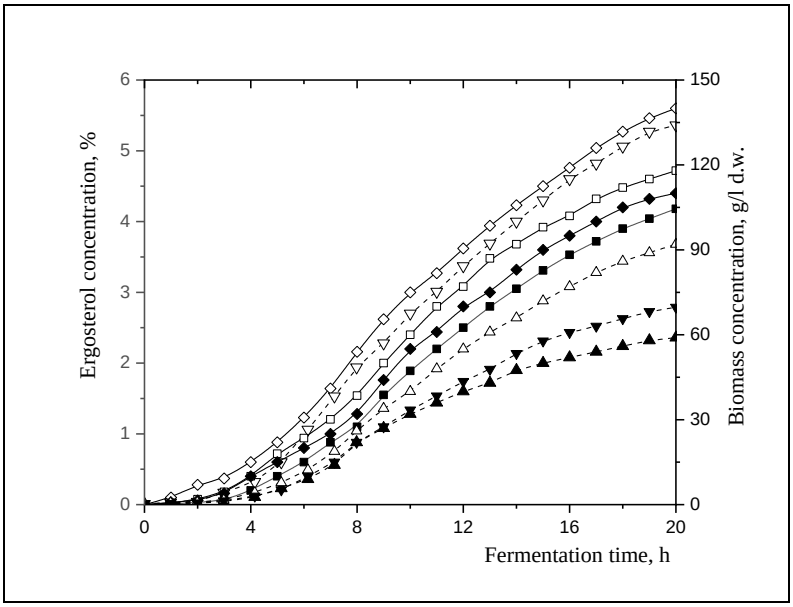

Figure 1. Variations of ergosterol and biomass concentrations during fed-batch fermentation process (ergosterol: □ - 0% vol. n-dodecane, ■ - 5% vol. n-dodecane, ◊ - 10% vol. n-dodecane, ♦ - 15% vol. n-dodecane; biomass: ▲- 0% vol. n-dodecane, Δ - 5% vol. n-dodecane, ∇ - 10% vol. n-dodecane, ▼ - 15% vol. n-dodecane).

The variation of ergosterol concentration inside the *S. cerevisiae* cells with the increase of air superficial velocity is not similar to that of biomass amount (Figure 2). Therefore, by increasing the air superficial velocity from 5 to 25 x 10^{-5} m/s in presence of n-dodecane, the maximum of ergosterol content is moved to higher aeration rates (for 5% vol. hydrocarbon, the maximum can be reached to over 25 x 10^{-5} m/s air superficial velocity, while for 10 and 15% vol. it corresponds to 21 x 10^{-5} m/s). This variation of ergosterol content inside the cells is due to the oxygen inhibitory effect, phenomenon which is more important at intense aeration and hydrocarbon volumetric fraction over 0.5.

The above discussed influences of biomass concentration, aeration rate, and n-dodecane volumetric fraction on the amount of ergosterol accumulated inside the yeast cells have been included in a mathematical expression which could be used for process optimization. This mathematical model was established by statistical analysis, using a factorial experiment of second order. Therefore, the real values of the process variables were chosen arbitrarily, Table 1 indicating their limits and coding.

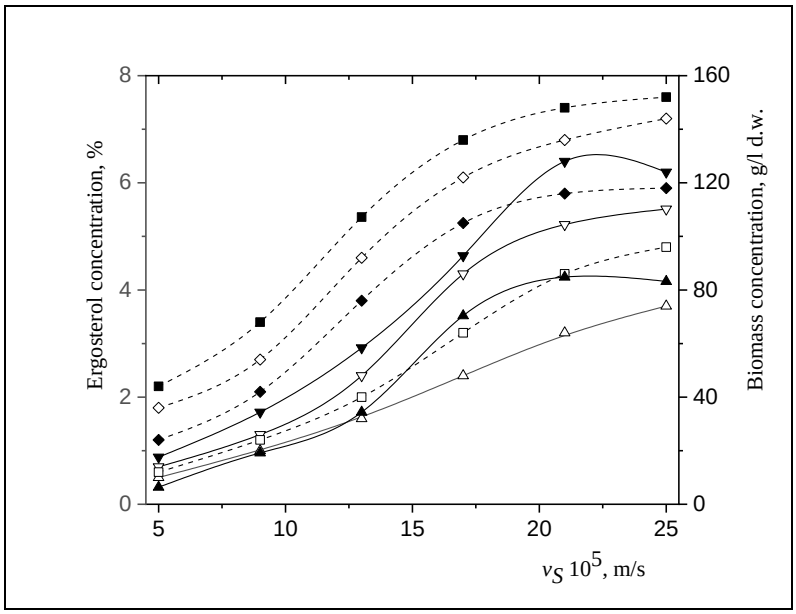

Figure 2. Variations of ergosterol and biomass concentrations with aeration rate in fed-batch fermentation process (ergosterol: Δ - 0% vol. n-dodecane, ▼ - 5% vol. n-dodecane, ∇ - 10% vol. n-dodecane, ▲ - 15% vol. n-dodecane; biomass concentration: □ - 0% vol. n-dodecane, ◊ - 5% vol. n-dodecane, ■ - 10% vol. n-dodecane, ♦ - 15% vol. n-dodecane).

Table 1. The limits and coding of process variables for modeling ergosterol production

Variable	Code	Variable level			Step
		-1	0	+1	
Biomass concentration, g/L d.w.	x_1	40	80	120	1
Air superficial velocity, m/s	x_2	15x10^{-5}	20x10^{-5}	25x10^{-5}	5x10^{-5}
n-Dodecane volumetric fraction, -	x_3	0.05	0.10	0.15	0.05

In order to settle the correlation between the ergosterol amount biosynthesized by yeasts, *Y*, and the above mentioned parameters, the following model of polynomial equation type has been proposed:

$$Y = b_0 + b_1 \cdot x_1 + b_2 \cdot x_2 + b_3 \cdot x_3 + b_{12} \cdot x_1 \cdot x_2 + b_{13} \cdot x_1 \cdot x_3 + b_{23} \cdot x_2 \cdot x_3 + \\ + b_{11} \cdot x_1^2 + b_{22} \cdot x_2^2 + b_{33} \cdot x_3^2 \quad (1)$$

where b_0,..., b_{33} are the regression coefficients.

The factorial experiment matrix of second order and the corresponding experimental values of ergosterol amount, Y_{exp}, are presented in Table 2.

Table 2. The experimental matrix

No. exp.	x_1	x_2	x_3	Y_{exp}, %
1.	-1	-1	-1	4.2
2.	1	-1	-1	9.5
3.	-1	1	-1	7.5
4.	1	1	-1	12.1
5.	-1	-1	1	4.9
6.	1	-1	1	7.7
7.	-1	1	1	5.1
8.	1	1	1	9.1
9.	-1	0	0	5.9
10.	1	0	0	9.8
11.	0	-1	0	3.4
12.	0	1	0	4.2
13.	0	0	-1	8.0
14.	0	0	1	5.7
15.	0	0	0	5.8
16.	0	0	0	5.7
17.	0	0	0	6.0

By means of the obtained data, the regression coefficients have been calculated using the following relations (BALABAN, 1993):

$$b_0 = \overline{Y}_{15-17}, b_j = \frac{\sum_{i=1}^{15} x_{ji} Y_i}{\sum_{i=1}^{15} x_{ji}^2}, b_{jk} = \frac{\sum_{i=1}^{15} x_{ji} x_{ki} Y_i}{\sum_{i=1}^{15} x_{ji}^2 x_{ki}^2}, b_{jj} = \frac{\sum_{i=1}^{15} x'_{ji} Y_i}{\sum_{i=1}^{15} \left(x'_{ji}\right)^2}, x'_{ji} = x_{ji}^2 - \frac{1}{15} \cdot \sum_{i=1}^{15} x_{ji}^2 \quad (2)$$

i = 1...15 number of experiments;
j = 1...3 number of variables.

The calculated values of regression coefficients are given in Table 3. For checking the normal results from the program center (experiments no. 15-17), the Q test was used (BALABAN, 1993). Therefore, the calculated Q values are:

$$Q = \frac{|a_1 - a_2|}{A} = 0.67 \quad (3)$$

where: a_1 - the uncertain value (6.0%);
a_2 - the closest to the uncertain value (5.8%);
A - the amplitude (difference between the most distant values: 0.30%).

Table 3. The values of regression coefficients for ergosterol production

Regression coefficient	b_0	b_1	b_2	b_3	b_{12}	b_{13}	b_{23}	b_{11}	b_{22}	b_{33}
Values	5.83	1.97	0.80	-1.12	0.10	-0.18	-0.18	1.83	-1.53	-1.22

For a certain threshold of 0.05, Q = 0.77 was found in literature (BALABAN, 1993). Because the calculated Q value is lower than the tabulated one, it could be concluded that the uncertain value of 6.0% is also a normal one. Consequently, all of the three values of ergosterol amount corresponding to the program center (experiments no. 15-17) can be considered for calculation.

Hence, the specific expression of regression equation is:

$$Y = 5.83 + 1.97 \cdot x_1 + 0.80 \cdot x_2 - 1.12 \cdot x_3 + 0.10 \cdot x_1 \cdot x_2 - 0.18 \cdot x_1 \cdot x_3 - 0.18 \cdot x_2 \cdot x_3 + 1.83 \cdot x_1^2 - 1.53 \cdot x_2^2 + 1.22 \cdot x_3^2 \quad (4)$$

The values of ergosterol amount, Y_{calc}, calculated by means of the mathematical expression (4) are given in Table 4.

The limits between which the values of parameter Y calculated with the regression equation (4) oscillate around the experimental values are established with the relationship (BALABAN, 1993):

$$Y_{calc_i} = Y_{\exp_i} \pm t \cdot S_{Yx}, \% \qquad (5)$$

The standard deviation S_{Yx}^2 was calculated as follows (BALABAN, 1993):

$$S_{Yx}^2 = \frac{\sum_{i=1}^{8} \left(Y_{\exp_i} - Y_{calc_i}\right)^2}{n - (k+1)} \qquad (6)$$

where n is the number of experiments and k the number of variables taking into account. The standard deviation for the modeled parameters is $S_{Yx}^2 = 0.0475$.

Table 4. The values of ergosterol amount calculated by means of the mathematical expression

No. exp.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
$\mathbf{Y_{calc}}$**, %**	4.0	9.5	7.4	11.9	5.0	7.5	5.2	8.9	5.7	9.6	3.5	4.3	8.2	5.9	5.83

The t values are given in the Tables for Student distribution (BALABAN, 1993). Therefore, for a confidence threshold of 0.05 and 15 experiments, t is 2.131. Consequently, the calculated amount of ergosterol oscillates around its experimental value into the following limits:

$$Y_{calci} = Y_{\exp_i} \pm 0.464, \% \qquad (7)$$

The individual influence of the considered factors on the parameter Y describing the ergosterol accumulation inside the *S. cerevisiae* cells is estimated by means of the value of the correlation coefficient, r_{Yx} (BALABAN, 1993):

$$r_{Yx_i} = \frac{\sum_{i=1}^{8} \left[x_i \cdot \left(Y_i - \overline{Y}\right)\right]}{\sqrt{\sum_{i=1}^{8} x_i^2 \cdot \sum_{i=1}^{8} \left(Y_i - \overline{Y}\right)^2}} \qquad (8)$$

The correlation coefficient indicates the nature of dependence between the process variables and the considered parameter Y. The determination coefficient, which is the square of correlation coefficient, represents the fraction of parameter Y variation that can be explained by variable x_i influences. For the studied fermentation process, the calculated values of the three determination coefficients are:

$$r_{Yx_1}^2 = 0.424 \qquad r_{Yx_2}^2 = 0.307 \qquad r_{Yx_3}^2 = 0.213$$

These values suggest that the considered variables control the production of ergosterol in 94.4% extent, while the rest of 5.6% could be attributed to the less important effects of other factors that were not taken into account, namely: glucose concentration, mixing intensity, impeller configuration, etc.

Conclusions

The production of ergosterol by *S. cerevisiae* cells depends strongly on the biomass amount and dissolved oxygen level inside the broth. The oxygen supply controls not only the yeast cells accumulation rate, but mainly the ergosterol biosynthesis, according to the related metabolic pathway which requires twelve molecules of oxygen for converting squalene into ergosterol. The aeration efficiency could be improved by adding oxygen-vectors inside the broths, in this study n-dodecane being used in this purpose. In the same time, the yeast growth and ergosterol production can be affected by the inhibitory effect induced at higher dissolved oxygen level.

By means of the statistical analysis and using factorial experiments of second order, the production of ergosterol by *S. cerevisiae* in a fed-batch bioreactor has been modeled. The mathematical expression describes the cumulated influences on biomass concentration, aeration rate, and n-dodecane volumetric fraction on the concentration of biosynthesized ergosterol. For the investigated fed-batch system, the considered variables control the ergosterol production in a 94.4% extent, the biomass concentration inducing the most important influence.

Conflict of Interest

The authors have no conflict of interest to declare.

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