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CORRELATION BETWEEN AERATION AND ERGOSTEROL PRODUCTION BY YEASTS

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Abstract

The present study represents an extension of ergosterol production by *Saccharomyces cerevisiae* fermentation processes using oxygen-vector by establishing the influence of the analyzed main factors, such as aeration efficiency and hydrocarbon concentration. The study has been developed for batch and fed-batch fermentation systems and has been focused on the variation of ergosterol content inside the yeast cells during the fermentation cycle in correlation with hydrocarbon volumetric fraction, C_{OV}, glucose concentration, C_G, and air superficial velocity, v_S. Moreover, the variation of ergosterol content has been discussed in relation to the oxygen mass transfer coefficient, k_La. The experimental results obtained in both fermentation systems were quantified in two mathematical correlations describing the influences of the mentioned main parameters on ergosterol concentration, C_E. These two equations have the general expression $C_E = \alpha \cdot C_{OV}{}^{\beta} \cdot C_G{}^{\gamma} \cdot v_S{}^{\delta}$ (α, β, γ, δ are coefficients and exponents), and offer a good agreement with the experiments, the average deviations being ±5.94% for batch fermentation and ±4.18% for fed-batchfermentation.

Key words: air superficial velocity, ergosterol, *n*-dodecane, oxygen-vector, *Saccharomyces cerevisiae*.

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1. Introduction

Ergosterol (ergosta-5,7,22-trien-3β-ol), also known as provitamin D2, is the precursor of vitamin D2 (ergocalciferol), because it is converted under UV radiation to this vitamin (Rajakumar et al., 2007). The palette of ergosterol medical applications can be completed especially with its antitumor and antifungal activity (Ellis, 2002; Roberts et al., 2003; Yazawa et al., 2000). The main role of this sterol in microbial or plant cells is maintaining the cellular membrane physical integrity and vital functions, namely permeability through cell membrane for nutrients or metabolic products, as well as to ensuring the normal activity of plasma-membrane proteins activity and, implicitly, the cellular cycle (Bard et al., 1993; Bloch, 1983; Parks and Casey, 1995). These functions of ergosterol are similar to those of cholesterol in mammalian cells.

The natural sources of ergosterol are mainly the yeasts (*Saccharomyces spp.*, *Candida spp.*). It can also be found in fungus (*Claviceps spp.*) or plants (orchids) (Bard et al., 1993; Bloch, 1983; Huang et al., 2011; Parks and Casey, 1995). In the yeast cells, ergosterol is accumulated in the plasma-membrane as free form, and in membrane lipids as esters with fatty acids (Shobayashi et al., 2005; Veen et al., 2003). Ergosterol is biosynthesized in yeasts through complex metabolic pathway, which involves specific enzymes for converting squalene into this sterol by multiple steps (Daum et al., 1998; Lees et al., 1999; Parks et al., 1999; Souza et al., 2011).

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The production of ergosterol by chemical synthesis has some important disadvantages, due to the multiple steps and high requirements of materials and energy, with a low final yield (Wu et al., 2012). Therefore, a suitable alternative for ergosterol production at larger-scale is represented by aerobic fermentation using *S. cerevisiae* on different substrates (glucose, by-products from agriculture or food industry), in batch or fed-batch operating systems (Blaga et al., 2018; Shang et al., 2006; Tan et al., 2003; Wu et al., 2012).

Regardless of the fermentation systems used, the previous studies concluded that aeration efficiency is one of the major parameters that have to be considered in fermentation operating program. This statement is explained by the mechanism of ergosterol biosynthesis proposed by Rosenfeld and Beauvoit (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 and Beauvoit, 2003). Therefore, the level of dissolved oxygen in the broths or the oxygen uptake rate not only limits the yeasts growth, but also represents one of the main parameters controlling the ergosterol biosynthesis yield.

Consequently, the aeration efficiency of the yeasts broths constitutes one of the decisive factors of ergosterol biosynthesis at a satisfactory level. Generally, the bioreactor capacity to generate high rate of oxygen diffusion from air to the broths, or dissolved oxygen transfer through the liquid phase to the microorganisms, is limited by its design and operational characteristics. However, as it was observed for the biosynthesis of single-cell protein on various water insoluble hydrocarbon substrates, the addition of a nonaqueous organic phase intensifies significantly the oxygen transfer from air phase to cells, without needing a supplementary increasing of impellers rotation speed (Caşcaval et al., 2006; Clarke et al., 2006; Dumont et al., 2006, Galaction et. al., 2014).

The oxygen solubility in these compounds, defined as oxygen-vectors, is from several to over thirty times higher than in aqueous broths. The main classes of oxygen-vectors tested in fermentations were hydrocarbons, perfluorocarbons, and oils (Caşcaval et al., 2006; Clarke et al., 2006; Da Silva et al., 2008; Dumont et al., 2006; Li et al., 2012; Xu et al., 2007). Besides their high ability to dissolve oxygen, oxygen-vectors have to exhibit no toxicity against the cultivated microorganisms, and could be consumed by microbial cells as supplementary sources of carbon and energy.

In our previous studies the addition of n-dodecane led to the increase of almost 50% of ergosterol concentration, this variation being reached for the fed-batch process (Blaga et al., 2018). The volumetric fraction of oxygen-vector corresponded to the maximum content of ergosterol accumulated inside the yeasts cells depends on the fermentation system in direct relation with the biomass concentration. Therefore, for the batch process, the maximum ergosterol amount (2% from the *S. cerevisiae* biomass) was reached for 5% vol. n-dodecane, while for the fed-batch process the maximum concentration of ergosterol (6%) corresponded to 10% vol. hydrocarbon.

The previous experiments were continued and developed by analyzing different levels of aeration rate and n-dodecane concentration as influencing factors on ergosterol production by *S. cerevisiae* in batch and fed-batch fermentations. The importance of the studied factors was underlined in the two fermentation systems by means of the mathematical correlations proposed.

The present work led to the optimization of the fermentation processes for the production of ergosterol, by analyzing the most important parameters, such as aeration rate and oxygen-vector concentration, and by proposing novel mathematical equations that allow predicting the values of ergosterol concentration inside the *S. cerevisiae* cells.

2. Materials and methods

The experiments were carried out in 2 L laboratory stirred bioreactor (Fermac, Electrolab), provided with computer-controlled and recorded parameters. The bioreactor mixing system consists of one turbine impeller and three baffles (Matran et. al., 2015). The bioreactor and impeller characteristics have been presented in the previous paper (Blaga et al., 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^{-1} (corresponding to air superficial velocity, v_S, of 5 – 25 x 10^{-5} m s^{-1}). The domain of impeller rotation speed was 100 - 500 rpm. The dissolved oxygen concentration has been calculated as percent from the saturation level, according to the oxygen probe calibration.

The fermentation was carried out comparatively in batch and fed-batch systems. In the experiments *S. cerevisiae* has been used. In order to obtain the inoculum, a plate culture (plate media: 20 g L^{-1} peptone, 20 g L^{-1} glucose, 10 g L^{-1} yeast extract, 12 g L^{-1} agar) of yeast cells has been grown at 30±1 °C for 20 h .). 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 an optimized medium consisting of 60 g L^{-1} glucose, 31.2 g L^{-1} yeast extract, 7.8 g L^{-1} ammonium sulfate, 3.7 g L^{-1} potassium dihydrogen phosphate, 3.1 g L^{-1} magnesium sulfate, 1.25 g L^{-1} calcium chloride, 0.4 g silicon oil in tap water. After sterilization at 121 ºC for 20 min, the medium was inoculated with 5% vol. of inoculum. For the feed-batch fermentation, 60 mL of 600 g L^{-1} glucose solution was added into the bioreactor every 30 min, in the purpose to maintain the glucose level at minimum 10 g L^{-1} (Blaga et al., 2018).

In both fermentation systems, the temperature was 30ºC. 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^{-1} at 20 °C, oxygen solubility $54.9 \cdot 10^{-3}$ g L^{-1} at 35 ºC and atmospheric air pressure) (Rols et al., 1990). The sterilized hydrocarbon was added into the bioreactor at the beginning of fermentation, its volumetric concentration into the broth varied between 0 and 15%. The values of oxygen transfer rate, quantified by means of oxygen mass transfer coefficient, $k_L a$,have been calculated using the static method previously described (Caşcaval et al., 2006).

For ergosterol extraction, 0.2 g dry cells have been treated with 10 mL alcoholic solution of potassium hydroxide obtained by dissolving 8 g potassium hydroxide into 32 mL 60% vol. alcoholic solution (Shang et al., 2006). The extraction occurred after 3 h at 80 °C. After cooling at the room temperature, 10 mL of petroleum ether were added, the mixture being stirred for 2 min with a vortex. From extract phase were taken 2 mL and subjected to evaporation and the quantitative determination of ergosterol concentration was performed by HPLC method (Dionex Ultimate 3000 system using a Lichrospher Si 100 column 250 x 4.6 mm, 5 µm). The mobile phase was consisting of a mixture of n-hexane and tetrahydrofuran with volumetric ratio 85:15 and flow rate 1.0 mL min^{-1} (Shang et al., 2006). The HPLC system was provided with PDA detector at 280 nm. The ergosterol content has been considered as percent from the biomass amount.

The ergosterol content inside the yeasts cells was considered at the fermentation time corresponding to the maximum concentration of ergosterol in relation to hydrocarbon content, glucose content or fermentation time (Blaga et al., 2018).

Besides ergosterol percent accumulated at the fermentation end, the variations of glucose, biomass, ethanol, and dissolved oxygen concentrations during the fermentation have been analyzed for the batch and fed-batch operating conditions. The analysis of glucose and alcohol was performed also through HPLC method, using the same system equipped with refractive index detector and a HyperRez carbohydrate column (300 x 7.7 mm, 8 µm), with water as mobile phase at 0.6 mL min^{-1} and column temperature of 80 °C.

The biomass variation was analyzed spectrophotometrically by measuring the turbidity at 660 nm (Shang et al., 2006). 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 4.47 and 5.37%.

3. Results and discussion

3.1. The effect of glucose concentration and hydrocarbon volumetric fraction on ergosterol production in batch fermentation system

Obviously, regardless of the oxygen-vector concentration inside the broth, glucose is rapidly consumed during the first 9 hours (Fig. 1). At n-dodecane concentration over 10% vol., the rate of substrate consumption is reduced. According to Fig. 1, the closest glucose concentration, C_G, values to those corresponding to the absence of n-dodecane were recorded for hydrocarbon volumetric fraction, C_{OV}, of 0.15. In this case, after 9 hours of fermentation, the unconsumed substrate reaches its highest concentration and remains at 5 g L^{-1} even after 14 hours of fermentation.

The difference between the variations of glucose consumption at oxygen-vector volumetric fractions below and over 0.10 is due to the effects of hydrocarbon on dissolved oxygen amount and yeasts metabolism. The addition of n-dodecane leads to the increase of oxygen concentration inside the broth and of its mass transfer coefficient, both with positive influence on *S. cerevisiae* cells growth and, implicitly, on glucose consumption rate.

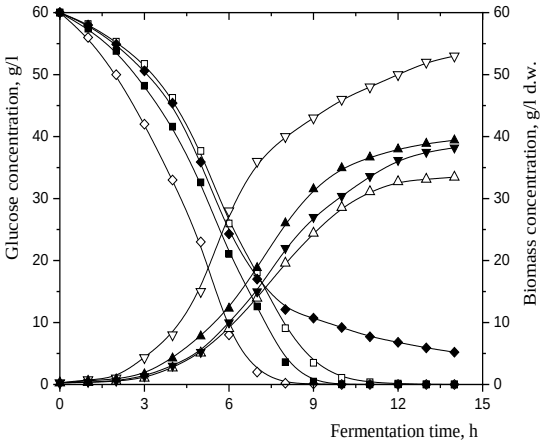

Fig.1. Variations of glucose and biomass concentrations during batch fermentation process (glucose: □ - 0% vol. n-dodecane, ■ - 5% vol. n-dodecane, ◊ - 10% vol. n-dodecane, ♦ - 15% vol. n-dodecane; biomass: Δ - 0% vol. n-dodecane, ▲ - 5% vol. n-dodecane, ∇ - 10% vol. n-dodecane, ▼ - 15% vol. n-dodecane).

However, at higher hydrocarbon volumetric fraction, namely 0.15, the substrate is consumed slower as the consequence of two possible phenomena: the inhibitory effect induced on *S. cerevisiae* growth at higher concentration of n-alkanes with over 8 carbon atoms chain (Bros, 1975), and the ability of yeasts to use n-dodecane as supplementary source of carbon and energy (Caşcaval et al., 2006).

The variation of biomass amount during the fermentation process is similar regardless of the n-dodecane volumetric fraction. From Fig. 1 it can be observed that the presence of hydrocarbon accelerates the accumulation of *S. cerevisiae* cells, mainly by facilitating the oxygen transfer from air phase to yeast cells and by contributing as additional source of carbon and energy. Similar to the influence of oxygen-vector of glucose consumption rate, the maximum concentration of yeast biomass is reached for 10% vol. n-dodecane, while the minimum one for 15% vol. n-dodecane, for the above mentioned reasons. Moreover, the role of n-dodecane as supplementary substrate allows *S. cerevisiae* biomass to growing also after the glucose is totally consumed (for fermentation without hydrocarbon, the yeasts concentration reached a constant level over 12 hours).

The level of dissolved oxygen concentration into the broth controls not only the yeast cells growth, but also the metabolic equilibrium between the utilization of glucose for energy production, simultaneously with production of carbon dioxide and water as metabolites, and alcohol biosynthesis. According to literature, the yeasts can be divided into "Crabtree-negative" yeasts, which are able to convert glucose to alcohol under anaerobic conditions only (e.g. *Candida spp.*, *Debaryomyces spp.*, *Kluyveromyces spp.*, *Pichia spp.*), and "Crabtree-positive" yeasts, which can combine the respiratory process with alcoholic fermentation also in presence of oxygen, at high glucose concentration (e.g. *Saccharomyces spp.*, *Brettanomyces spp.*, *Schizosaccharomyces spp.*) (Pfeiffer and Morley, 2014; Rosenfeld and Beauvoit, 2003).

The previous studies emphasized that the variation of oxygen concentration is contrary to that of glucose one during the fermentation cycle, the n-dodecane addition attenuating the significant decrease of dissolved oxygen amount recorded in the first 9 hours (Blaga et al., 2018). The positive influence of hydrocarbon on oxygen transfer rate from air bubble to liquid phase was diminished at the end of fermentation, the level of oxygen concentration for the system with n-dodecane becoming similar to that without this hydrocarbon. The reduction of the magnitude of oxygen-vector effect on aeration efficiency was considered to be the results of three possible phenomena occurring at higher biomass amount: the increase of broth viscosity by accumulating yeast cells, the consumption of hydrocarbon as alternative substrate, and the blockage of n-dodecane droplets surface available for oxygen transfer between the organic phase and broth by cells adsorption due to the yeast cells affinity for hydrocarbon (Blaga et al., 2018; Caşcaval et al., 2006). This additional resistance to oxygen transfer can be amplified by the adsorption of cells-droplets associations to the air bubbles, thus reducing the bubble surface and generating a new barrier against the oxygen diffusion from the air phase to the cells.

The metabolism of *S. cerevisiae* is included in "Crabtree-positive" group, this yeast being able to biosynthesize ethanol from glucose in aerobic conditions. In these circumstances, *S. cerevisiae* produces ethanol both in absence and presence of n-dodecane (Fig. 2).

The evolution of alcohol concentration during the fermentation process depends on the oxygen-vector amount added into the broth. Therefore, Fig. 2 indicates that for hydrocarbon volumetric fraction up to 0.05, the ethanol concentration increases, reaches a maximum value after 9 hours from the fermentation start, decreasing then. The maximum amount of alcohol corresponds to the end of the exponential growth of yeast cells, implicitly to the consumption of almost entire glucose amount (85% of glucose amount consumed in the system without n-dodecane, 95% with 5% vol. n-dodecane).

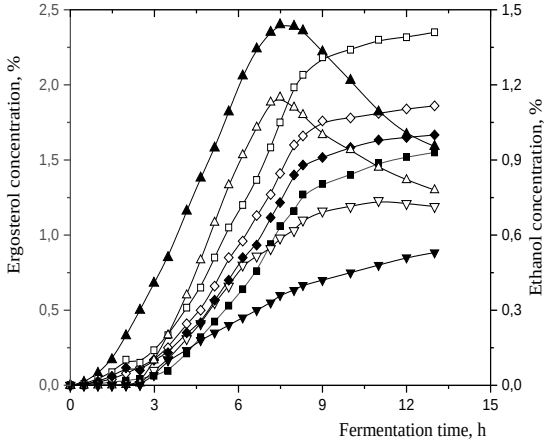

Fig. 2. Variations of ergosterol and ethanol concentrations during batch fermentation process (ergosterol: ■ - 0% vol. n-dodecane, □ - 5% vol. n-dodecane, ◊ - 10% vol. n-dodecane, ♦ - 15% vol. n-dodecane; ethanol: ▲ - 0% vol. n-dodecane, Δ - 5% vol. n-dodecane, ∇ - 10% vol. n-dodecane, ▼ - 15% vol. n-dodecane).

The lower concentration of dissolved oxygen into the broth without n-dodecane allows accumulate higher amount of alcohol compared to the fermentation system containing 5% vol. hydrocarbon. In both cases, the decrease of ethanol concentration over 9 hours of fermentation can be attributed to its utilization as additional substrate. The importance of this process is attenuated in presence of n-dodecane, which compensates partially the very low concentration of the main substrate, glucose, over 9 - 10 hours of fermentation.

The increase of oxygen-vector amount into the broth leads to the significant diminution of the alcohol concentration. Moreover, by increasing the volumetric fraction of oxygen-vector over 0.05, the variation of ethanol concentration differs more and more from the above discussed one. From Fig. 2 it can be seen that for 10% vol. n-dodecane, the concentration of alcohol increases during the first 9 hours of fermentation and remains at an almost constant level towards the end of fermentation. At 15% vol. hydrocarbon, the ethanol concentration increase continuously during the entire fermentation cycle.

This difference between the variations of alcohol concentrations for n-dodecane volumetric fraction below and, respectively, over 0.05 is the result of the higher amount of hydrocarbon in the second case, which replaces glucose, already consumed, and alcohol as substrates in the metabolic pathway of yeasts.

The variation of ergosterol concentration inside the *S. cerevisiae* cells can be generally related to the variations of biomass and, implicitly, glucose concentrations into the broths, as well as to the amount of biosynthesized ethanol (Fig. 2). All these parameters are controlled by the oxygen concentration, which depends in turn on the volumetric fraction of hydrocarbon. However, as can be observed from Fig. 1 and 2, the dependence between the biomass concentration and n-dodecane amount is not perfectly similar to that between the ergosterol concentration and n-dodecane amount.

Regardless of oxygen-vector concentration, the ergosterol amount strongly increases simultaneously with the significant growth of the yeast biomass (period which is limited between 2 - 3 and 8 - 9 hours from the fermentation start), this variation being correlated with the rapid consumption of glucose and oxygen. Obviously, due to the increased demand of oxygen, the concentration of ergosterol is higher in presence of oxygen-vector compared to the fermentation system without hydrocarbon. The rate of ergosterol accumulation is reduced over 9 hours, not only due to the glucose consumption, but also due to the negative effects induced by hypoxia and ethanol production (Pfeiffer and Morley, 2014). The magnitude of these effects is more important in absence of n-dodecane, as the result of higher amount of biosynthesized alcohol.

By analyzing the ergosterol production in systems with n-dodecane, it was established that the highest amount of ergosterol (2.3% at 14 hours of fermentation) was reached for 5% vol. n-dodecane, while the lowest concentration (1.6% at same conditions) for 15% vol. hydrocarbon (in absence of n-dodecane, the maximum ergosterol amount was 1.3% at the fermentation end) (Fig. 2). According to Fig. 1, the maximum rates of biomass growth and glucose consumption correspond to 10% vol. n-dodecane. Therefore, it can be concluded that the value of oxygen-vector concentration related to the maximum accumulation rate of *S. cerevisiae* cells does not lead to the maximum amount of ergosterol, being twice as higher than that corresponded to 2.3% ergosterol. This non-concordance is the cumulated result of the inhibitory effects induced both by higher hydrocarbon concentration and, especially, higher oxygen concentration, because oxygen acts as limiting substrate in the yeast metabolic pathway for ergosterol biosynthesis (Rosenfeld and Beauvoit, 2003).

The main contribution of n-dodecane to ergosterol production by *S. cerevisiae* is to intensify the oxygen transfer from air bubble to yeast cells. In this context, Fig. 3 indicates the significant increase of k_La by increasing the n-dodecane concentration in the considered domain of variation.

For example, at the $v_s$of at 5 x 10^{-5} m s^{-1}, the oxygen transfer rate inside the broth containing 15% vol. hydrocarbon is for 15 times greater than for the system without hydrocarbon. For a given biomass concentration value, this difference is strongly amplified by increasing the aeration rate, as the consequence of increasing turbulence promoted by aeration. The intensified turbulence induces the disruption of the bubble-cells-droplets association and generates free surfaces of bubbles and hydrocarbon droplets that are able to facilitate oxygen transfer (Cașcaval et al., 2006). The magnitude of effect is amplified at higher amount of oxygen-vector, due to the higher reserve of oxygen inside the hydrocarbon droplets. Consequently, at 15% vol. n-dodecane, k_La becomes for six times greater than k_La recorded at 0% vol. hydrocarbon (Fig. 3).

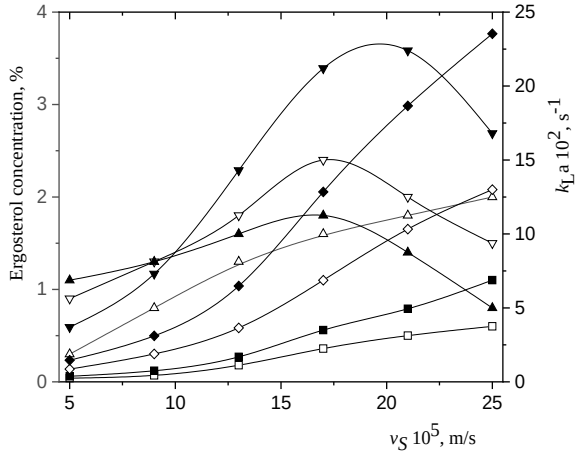

Fig. 3. Variations of ergosterol concentration and k_La with aeration rate in batch fermentation process (ergosterol: Δ - 0% vol. n-dodecane, ▼ - 5% vol. n-dodecane, ∇ - 10% vol. n-dodecane, ▲ - 15% vol. n-dodecane; k_La: □ - 0% vol. n-dodecane, ■ - 5% vol. n-dodecane, ◊ - 10% vol. n-dodecane, ♦ - 15% vol. n-dodecane).

In the context of above discussed influence of n-dodecane amount on ergosterol production, Fig. 3 indicates a particular variation of ergosterol concentration by increasing the aeration rate. Thus, for air superficial velocity below 9 x 10^{-5} m s^{-1}, the ergosterol concentration increase by varying the oxygen-vector volumetric fraction from 0 to 0.15, in concordance with the role of hydrocarbon in accelerating the oxygen transfer. The intensification of aeration leads to a continuous increase of ergosterol production only for the system without n-dodecane. For the broths containing hydrocarbon, the increase of aeration rate leads initially to the increase of ergosterol concentration, followed by its decreasing. The maximum amount of ergosterol produced by yeast cells corresponds to 21 x 10^{-5} m s^{-1} air superficial velocity for 5% vol. n-dodecane, and to 17 x 10^{-5} ms^{-1} for 10 and 15% vol. hydrocarbon (Fig. 3). In the same time, for aeration rate over 9 x 10^{-5} m s^{-1}, the most

important production of ergosterol was recorded for 5% vol. n-dodecane.

These results confirm the decisive role of inhibition phenomenon generated at higher oxygen concentration. By intensifying the aeration, the threshold for inducing the inhibitory effect of oxygen is reached more rapidly for n-dodecane concentration over 10% vol. and becomes very important at higher aeration rate. For this reason, at 25 x 10^{-5} m s^{-1} air superficial velocity, the ergosterol production for the fermentation without hydrocarbon exceeds that obtained in the fermentation with 10 and 15% vol. hydrocarbon. At this value of aeration rate and given experimental conditions, the maximum concentration of ergosterol was recorded for 5% vol. n-dodecane, due to the less important inhibitory effects of hydrocarbon and oxygen, compared to the other considered hydrocarbon volumetric fractions, and to its utilization as additional substrate, compared to the system without hydrocarbon.

3.2. The effect of glucose concentration and hydrocarbon volumetric fraction on ergosterol production in fed-batch fermentation system

The glucose was additionally added into the broth when its concentration reached a value close to 10 g L^{-1}. Regardless of n-dodecane content into the fermentation broth, the biomass concentration was significantly increased using the fed-batch system compared to the batch one, mainly as the result of the extension of period corresponding to the biomass exponential growth. Fig. 4 indicates clearly that the amount of biomass accumulated over 14 hours of fermentation is for about 1.4 - 1.9 times greater than in batch fermentation. The difference between the *S. cerevisiae* concentrations in the two fermentation systems becomes more important by increasing the n-dodecane concentration from 0 to 10% vol., due to the increase of dissolved oxygen concentration.

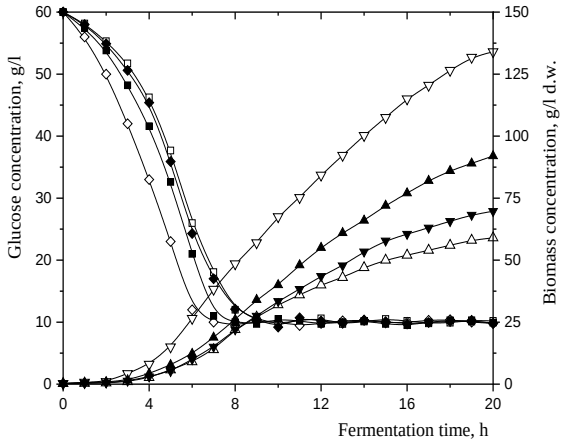

Fig. 4. Variations of glucose and biomass concentrations during fed-batch fermentation process (glucose: □ - 0% vol. n-dodecane, ■ - 5% vol. n-dodecane, ◊ - 10% vol. n-dodecane, ♦ - 15% vol. n-dodecane; biomass: Δ - 0% vol. n-dodecane, ▲ - 5% vol. n-dodecane, ∇ - 10% vol. n-dodecane, ▼ - 15% vol. n-dodecane).

At 15% vol. hydrocarbon, the ratio between the yeasts concentration recorded in the fed-batch fermentation and that for the batch fermentation is 1.4, being similar to the value obtained in absence of oxygen-vector. Moreover, 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, being rather similar to the value reached in absence of hydrocarbon. Because the glucose concentration level is maintained at a satisfactory constant level, this diminishing the potential use of n-dodecane as supplementary substrate by yeasts; the probability to generate the substrate inhibitory effect at high amount of n-alkanes is low. Consequently, the low production on biomass recorded for 15% vol. n-dodecane could be explained by the inhibitory effect induced at higher oxygen concentration.

As in the batch system, the presence of n-dodecane leads to the acceleration of glucose consumption rate. In the fermentation system without oxygen-vector, the glucose addition was necessary over the first 9 - 10 hours, while for the fermentation with hydrocarbon over 6 to 9 hours. The highest rate of glucose consumption was reached for a volumetric fraction of n-dodecane of 0.10, while the lowest one for 0 and 0.15 volumetric fractions. In this circumstance, it can be assumed that the effect of low concentration of dissolved oxygen is similar to that of high oxygen concentration. For all experimented hydrocarbon contents into the broth, ethanol is continuously produced during the entire duration of fed-batch fermentation by providing constantly the glucose level around 10 g L^{-1} and, implicitly, by avoiding the alcohol consumption as additional substrate (Fig. 5). In these cases, the alcohol concentration over 20 hours of fermentation became for 1.5 to 2.2 times higher than the maximum one reached in batch system for each considered oxygen-vector volumetric fraction (the most significant increase was recorded in the absence of n-dodecane, the difference being diminished from 5 to 15% vol. n-dodecane).

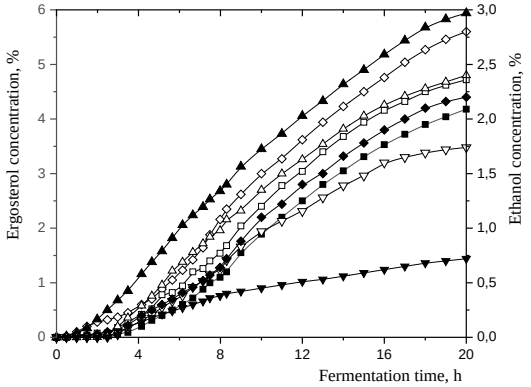

Fig. 5. Variations of ergosterol and ethanol concentrations during fed-batch fermentation process (ergosterol: ■ - 0% vol. n-dodecane, □ - 5% vol. n-dodecane, ◊ - 10% vol. n-dodecane, ♦ - 15% vol. n-dodecane; ethanol: ▲ - 0% vol. n-dodecane, Δ - 5% vol. n-dodecane, ∇ - 10% vol. n-dodecane, ▼ - 15% vol.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

Moreover, as the result of the increase of the relative importance of aerobic metabolism of *S. cerevisiae* and, therefore, of the amplification of the inhibitory effect magnitude on alcohol biosynthesis, the ethanol concentration over 20 hours is reduced for about 4 times by varying the hydrocarbon volumetric fraction from 0 to 0.15.

Although the ergosterol is continuously biosynthesized during the fermentation cycle, the rate of this sterol production depends on the presence and concentration of n-dodecane (Fig. 5). In the case of fed-batch fermentation, the maximum ergosterol concentration corresponds to 10% vol. n-dodecane, not to 5% vol. as in the batch process. In the fed-batch system, the higher concentration of *S. cerevisiae* biomass diminishes the positive role of n-dodecane on aeration efficiency by increasing the broth apparent viscosity and the amplitude of the interactions of adsorption type between the hydrocarbon droplets - yeast cells associations and air bubbles. For this reason, in the fed-batch fermentation, higher oxygen-vector concentration is required for reaching the maximum ergosterol content inside the yeast cells. Over this hydrocarbon volumetric fraction, its inhibitory effect on *S. cerevisiae* growth and "Crabtree-positive" metabolism lead to the reduction of ergosterol production.

Moreover, due to the maintaining of glucose concentration at a constant level in the fed-batch system, the ergosterol content inside the *S. cerevisiae* cells for 5% vol. hydrocarbon exceeds that produced in similar conditions but in batch fermentation (in presence of 5% vol. n-dodecane, the ergosterol concentration in *S. cerevisiae* cells in fed-batch-system was for 2.2 times higher than that obtained in batch fermentation). In this case, it can be observed that the negative influence of alcohol accumulation inside the broth on ergosterol biosynthesis is counteracted by providing constantly the glucose, as the consequence of the higher concentration of yeasts biomass.

As the result of the inhibitory effects induced at higher amounts of n-alkanes, whose consumption as additional substrate by yeasts is diminished by continuous glucose feeding, and of dissolved oxygen as limiting substrate, the level of ergosterol production for 15% vol. n-dodecane is close to that recorded for the absence of the hydrocarbon.

From Fig. 6 it can be observed that k_La increases for several times by increasing the volumetric fraction of n-dodecane from 0 to 0.15, this effect being amplified at higher air superficial velocity (at $5x10^{-5}$ m s^{-1}, the ratio between value of k_La corresponding to 15% vol. hydrocarbon and that recorded for 0% vol. hydrocarbon is 4.4, becoming 6.2 at 25 x 10^{-5} m s^{-1}).

As it was previously concluded, the constant feeding with glucose allows to reaching higher yeast cells amount in the fed-batch system (after 14 hours of fermentation, the concentration of *S. cerevisiae* biomass obtained in fed-batch bioreactor is almost double compared to that reached in batch bioreactor). For this reason, the influence of n-dodecane in intensifying the oxygen transfer is diminished in fed-batch fermentation compared to the batch one. Therefore, the oxygen transfer coefficient is for 1.2 to 1.8 times higher in batch fermentation, the difference becoming less significant by increasing the aeration rate (Fig. 6).

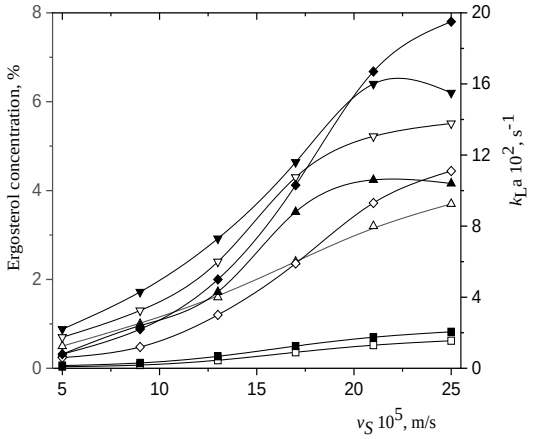

Fig. 6. Variations of ergosterol concentration and k_La with aeration rate in fed-batch fermentation process (ergosterol: Δ - 0% vol. n-dodecane, ▼ - 5% vol. n-dodecane, ∇ - 10% vol. n-dodecane, ▲ - 15% vol. n-dodecane; k_La: □ - 0% vol. n-dodecane, ■ - 5% vol. n-dodecane, ◊ - 10% vol. n-dodecane, ♦ - 15% vol. n-dodecane).

The above analysed comparisons between the batch and fed-batch systems from the viewpoint of efficiency of oxygen transfer are the consequence of the formation of bubble-cells-droplets associations, phenomenon which is more pronounced in the fermentation containing higher amount of *S. cerevisiae* biomass (Cașcaval et al., 2006). Thus, the interfacial area between air bubbles and broth blocked by the adsorbed associations between hydrocarbon droplets and yeast cells is more extended in fed-batch fermentation system. This conclusion is supported by the effect of aeration intensification, which leads to the increase of turbulence and, consequently, to the disruption of these associations, generating free surfaces of bubbles and hydrocarbon droplets able to facilitate the oxygen transfer. The magnitude of positive influence of increasing the aeration rate on reducing the difference between the values of k_La for the batch and fed-batch systems becomes more important at greater content of hydrocarbon, due to the higher reserve of oxygen inside the n-dodecane droplets.

Moreover, although by respecting a constant level of glucose the alternative consumption of hydrocarbon is avoided in the fed-batch fermentation, the higher yeasts concentration reached in fed-batch fermentation induces another stronger limitation on oxygen diffusion from air bubbles to yeast cells in presence of n-dodecane, namely the higher apparent viscosity of broth.

The variation of ergosterol concentration inside the yeast cells with the increase of air superficial velocity is the result of the effects valid also for the

batch fermentation, the dependences being similar (Fig. 6). Besides this similarity, by varying the air superficial velocity from 5 to 25 x 10^{-5} m s^{-1}, Fig. 6 suggests that the maximum of ergosterol concentration is moved to higher aeration rates in presence of n-dodecane (for 5% vol., the maximum is possible to be reached over 25 x 10^{-5} m s^{-1} air superficial velocity, while for 10 and 15% vol. it corresponds to 21 x 10^{-5} m s^{-1}).

Similar to the batch fermentation system, it can be assumed that the above discussed variations of ergosterol content inside the cells are controlled by the oxygen inhibitory effect, phenomenon more pronounced at intense aeration and hydrocarbon volumetric fraction over 0.5. However, in the case of fed-batch process, which allows to reaching higher amount of *S. cerevisiae* biomass, the inhibition induced by oxygen is attenuated due to the blockage of the interfacial area between air bubbles and aqueous phase by the adsorbed associations hydrocarbon droplets - yeasts. Consequently, for inducing the oxygen inhibitory effect more intense aeration is required. The same effect is responsible for closer values of ergosterol content recorded for 5% vol. and 10% vol. n-dodecane at 25 x 10^{-5} m s^{-1} or over this level of air superficial velocity.

3.3. Mathematical correlations for ergosterol production in presence of oxygen-vectors

By means of the experimental data obtained for the analyzed fermentation systems for ergosterol production, two mathematical correlations which describe the influence of oxygen-vector concentration, glucose concentration, air superficial velocity, and fermentation time on ergosterol concentration inside S. cerevisiae cells have been proposed for the batch and fed-batch processes. The general expression of the proposed equations is (Eq. 1):

$$C_E = \alpha \cdot C_{OV}^{\ \beta} \cdot C_G^{\ \gamma} \cdot v_S^{\ \delta} \quad (1)$$

where:
C_E is the ergosterol concentration (% related to yeast cells weight);
C_{OV} is the n-dodecane volumetric fraction (-);
C_G is the glucose concentration (kg m^{-3});
v_S is the air superficial velocity (m s^{-1});
α, β, γ, δ are coefficients and exponents, the coefficient γ value depending on fermentation time.

For the calculation of the α, β, γ, and δ coefficients values, by means of the multiregression method, MATLAB software was used. Thus, the following correlations have been obtained (Eq. 2 and Eq. 3):

- batch fermentation

$$C_E = 2.57 \cdot 10^{-2} \cdot \frac{C_{OV}^{\ 0.93} \cdot v_S^{\ 0.16}}{C_G^{\ 6.4 \cdot 10^{-2} \cdot t_F}} \quad (2)$$

- fed-batch fermentation

$$C_E = 0.87 \cdot \frac{C_{OV}^{\ 0.63} \cdot v_S^{\ 0.15}}{C_G^{\ 4 \cdot 10^{-2} \cdot t_F}} \quad (3)$$

where t_F is the fermentation time (h).

The proposed equations (Eq.2, Eq. 3) are valid for the following domains of studied parameters: $C_{OV} \in$ (0, 0.15], $C_G \in$ (0, 60], $v_S \in$ [5 x 10^{-5}, 25 x 10^{-5}] and offer a good agreement with the experimental data, the average deviations being ±5.94% for batch fermentation and ±4.18% for fed-batchone.

The previous analysed influences of the considered parameters and their importance are suggested by the sign and magnitude of the corresponding exponents. As it was above discussed, the hydrocarbon volumetric fraction exhibits a more significant positive influence in the case of batch fermentation, as the result of lower concentration of substrate, while the influence of superficial air velocity is similar in both fermentation systems.

4. Conclusions

The production of ergosterol by *S. cerevisiae* cells is strongly depended on the dissolved oxygen content into the fermentation broth. For this reason, the influence of aeration efficiency on ergosterol accumulation inside the yeast cells has been studied in direct relation to the presence and amount of n-dodecane, as oxygen-vector. The role of aeration and, implicitly, oxygen concentration, has been analysed by means of the air superficial velocity, oxygen transfer rate, in different fermentation operating conditions (batch and fed-batch systems, air superficial velocity) and broths characteristics (n-dodecane concentration, glucose concentration, biomass concentration).

Regardless of n-dodecane volumetric fraction, the use of fed-batch operating system led to an increase of ergosterol concentration for over two times, this difference from the batch fermentation becoming more pronounced in presence of n-dodecane. The blockage of the interfacial area of air bubbles by the adsorption of hydrocarbon droplets - yeast cells associations exhibits a major role on limiting the oxygen content into the broth and, consequently, on ergosterol production. This negative effect is more important than the inhibitory phenomena induced by higher concentrations of hydrocarbon or oxygen. Therefore, the maximum ergosterol amount was reached for 5% vol. n-dodecane in batch process, but was move to 10% vol. hydrocarbon for the fed-batch one. Moreover, the values of air superficial velocity corresponding to the maximum ergosterol production have been moved to superior levels by changing the operating condition from batch to fed-batch one (the maximum ergosterol concentrations have been reached for 17 to 21x10^{-5} m s^{-1} in batch system, while for the fed-batch fermentation for 21 to over 25 x 10^{-5} m s^{-1}).

Both results are consequences of the higher yeast cells concentration in the fed-batch process, which partially counteracted the positive influence of oxygen-vector or of the turbulence promoted at higher aeration rate.

By means of the experimental data, two mathematical correlations have been proposed for batch and fed-batch fermentation systems. These equations allow to predicting the values of ergosterol concentration inside the *S. cerevisiae* cells on the basis of the above discussed influences. They offer a good concordance with the experimental results; the average deviations varying between ±4.18% for fed-batchfermentation and ±5.94% for batch one.

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