Biochemical Engineering Journal 131 (2018) 70–76

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

# Enhancement of ergosterol production by *Saccharomyces cerevisiae* in batch and fed-batch fermentation processes using *n*-dodecane as oxygen-vector

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ARTICLE INFO

*Article history:*
Received 5 September 2017
Received in revised form 7 December 2017
Accepted 12 December 2017
Available online 13 December 2017

*Keywords:*
Ergosterol
S. cerevisiae
Oxygen-vector
*n*-Dodecane
Ethanol
Fermentation

ABSTRACT

The effect of *n*-dodecane used as oxygen-vector in batch and fed-batch fermentations of *S. cerevisiae* for producing ergosterol has been comparatively investigated. Regardless of *n*-dodecane concentration, the results indicated the increase for about three times of ergosterol content inside the yeasts cells in fed-batch process. In both fermentation systems, by adding the oxygen-vector, the ergosterol concentration increased with over 50%. The oxygen-vector concentration corresponding to the maximum level of ergosterol depended mainly on biomass concentration, being 5% vol. for batch fermentation, and 10% vol. for fed-batch one. The increase of biomass concentration in the fed-batch process partially affected the positive influence of oxygen-vector, effect that became less significant only for hydrocarbon concentration over 10% vol. The inhibitory phenomenon induced at higher concentration of hydrocarbon also limits the positive influence of oxygen-vector, but this effect was partially counteracted in the fed-batch process due to the higher amount of yeasts biomass produced.



## 1. Introduction

Ergosterol (ergosta-5,7,22-trien-3β-ol, Fig. 1) was firstly isolated from fungal species *Claviceps*, but it was also found in yeasts and few herbs [1–4]. Similarly to the cholesterol in mammalian cells, this sterol plays the main role in ensuring the cellular membrane integrity and controlling its normal functions as fluidity or permeability and transport, as well as the plasma-membrane proteins activity and cellular cycle [1–3].

In the yeasts cells, ergosterol is encountered in membranes, being stored in its free form in the plasma-membrane, and as fatty acids esters in lipids [5,6]. The mechanism of ergosterol production in yeasts is complex and was the subject of multiples studies which led to a rather complete view on this sterol biosynthesis pathway. Specific enzymes are involved in the ergosterol biosynthesis by catalyzing the conversion of squalene to lanosterol, zymosterol, episterol, and finally to ergosterol [7–10].

Ergosterol is the precursor of ergocalciferol, also known as vitamin D2, being converted into it *via* viosterol by exposing to UV light [11]. For this reason, ergosterol is known as provitamin D2.

**Nomenclature**

| | |
|---|---|
| d | Impeller diameter (mm) |
| d' | Impeller diameter (mm) |
| D | Bioreactor diameter |
| h | Distance from the stirrer to the bioreactor bottom (mm) |
| H | Bioreactor height (mm) |
| l | Impeller blade length (mm) |
| l' | Oxygen electrode immersed length (mm) |
| R | Ratio between the ergosterol concentrations in fed-batch and batch fermentations |
| s | Baffle width (mm) |
| Y | Product yield factor related to glucose (g ergosterol/g glucose) |
| w | Impeller blade height (mm) |

Recently, other medical applications, as antitumor agent or specific target for antifungal compounds, have been developed [12–15].

Due to the presence of an asymmetric center in ergosterol molecule, its chemical synthesis is complicated and requires many steps with high materials and energy consumptions, while the

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https://doi.org/10.1016/j.bej.2017.12.010
1369-703X/

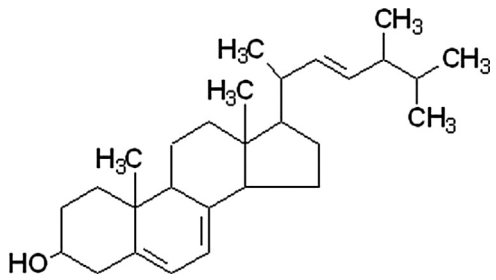


**Fig. 1.** Chemical structure of ergosterol.

yield is low [16]. Consequently, the aerobic fermentation processes became the most attractive methods for ergosterol production. Among various potential producers of ergosterol, different strains of *S. cerevisiae* have been preferred, especially due to their easier manipulation and the better knowledge on this sterol biosynthesis pathway [10]. The literature indicated that this sterol was produced by batch or fed-batch fermentations, on glucose or corn hydrolysis substrates, using different programs for substrate or oxygen feeding [6,16–18]. From these studies it was concluded that one of the main parameters which control the ergosterol yield is the level of dissolved oxygen in the broths or the oxygen uptake rate.

Therefore, the oxygen supply into 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 of dissolved oxygen transfer through the liquid phase to the microorganisms, depends on 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 could induce the significant increase of oxygen transfer rate from air to microorganisms, without needing a supplementary intensification of mixing [19–21]. The oxygen solubility in these compounds, called oxygen-vectors, is from several to over thirty times higher than in water. The main classes of oxygen-vectors tested in fermentations were hydrocarbons, perfluorocarbons, and oils [19–24]. Besides their high ability to dissolve oxygen, oxygen-vectors have to exhibit no toxicity against the cultivated microorganisms, and could be consumed as supplementary substrates (sources of carbon and energy).

Our previous studies on oxygen transfer inside *S. cerevisiae* broths indicated that the addition of *n*-dodecane led to the increase of oxygen mass transfer coefficient for up to 5 times [20]. The magnitude of this effect depends on the hydrocarbon concentration and specific power input.

On the basis of these results concerning the positive influence of *n*-dodecane addition on oxygen transfer rate and of the relation between the oxygen transfer efficiency and the amount of produced ergosterol, these experiments are aimed to investigate the possible positive effect of this oxygen-vector on ergosterol biosynthesized by *S. cerevisiae*. In this purpose, the influence of *n*-dodecane will be analyzed comparatively for batch and fed-batch fermentation systems related to the glucose feeding.

## 2. Materials and method

### 2.1. Bioreactor and operating parameters

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. The bioreactor and impeller characteristics are given in Table 1.

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 5 l $h^{-1}$. The rotation speed was maintained at 300 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 both fermentation systems, the temperature was 30 °C. The pH-value was maintained at 5.4, being automatically adjusted by addition of 25% ammonia solution.

### 2.2. Strain and medium

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 [25]) 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° C for 20 min, the medium was inoculated with 5% vol. 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 $l^{-1}$.

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 °C and atmospheric air pressure [26]). The sterilized hydrocarbon has been added into the bioreactor at the beginning of fermentation, its volumetric concentration into the broth varied between 0 and 15%.

### 2.3. Measurement and analysis methods

The values of oxygen transfer rate, quantified by means of $k_La$, have been calculated using the static method previously described [20].

For ergosterol extraction from biomass, 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 [18]. The extraction occurred 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. 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 × 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 $min^{-1}$ [18]. The HPLC system was provided with PDA detector at 280 nm. The ergosterol content has been considered as percent from the biomass amount.

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 × 7.7 mm, 8 μm), with water as mobile phase at 0.6 ml $min^{-1}$. The column temperature was 80 °C.

The biomass variation was analyzed spectrophotometrically by measuring the turbidity at 660 nm [18].