Acta Alimentaria 53 (2024) 2, 281–291
DOI: 10.1556/066.2024.00034

AKADÉMIAI KIADÓ

Stress modulation strategies in *Kluyveromyces marxianus*: Unravelling the effects of shear force and aeration for enhanced specific ergosterol production

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ORIGINAL RESEARCH PAPER

Received: February 6, 2024 • Accepted: May 9, 2024
Published online: June 4, 2024
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ABSTRACT

Ergosterol, as a precursor for synthesising useful molecules like vitamin D_2, possesses significant physiological functions in both fungal and human systems. In fungi, ergosterol plays a crucial role in stress responses. In contrast to *Saccharomyces cerevisiae* yeast, the changes in specific ergosterol content of *Kluyveromyces marxianus* under various stress conditions are less known. This study investigated how ergosterol content changes in response to different stress factors. Carbon to nitrogen (C/N) ratio was examined using experimental design. The effects of aeration and shear force beside constant overall volumetric mass transfer coefficient (KLa) were examined. Cell growth and specific ergosterol content were investigated using ethanol stress during a two-stage fermentation. Based on the results, contradictory settings regarding C/N ratio and shear force were found to be favourable for cell growth and specific ergosterol content. However, increased aeration consistently elevated specific ergosterol content and favoured cell growth as well (2.5-fold and 1.5-fold, respectively). In *K. marxianus* fermentations, higher ergosterol yield can be achieved through a two-stage fermentation (138.9 mg L^{-1} compared to 52.9 mg L^{-1}), where the first stage provides favourable conditions for cell growth, and the second stage involves stress (beneficial for ergosterol production) conditions. Conclusions drawn from the two-stage fermentation results suggest that early transitioning of cell growth to the second phase will not result higher adaption and specific ergosterol content compared to the transition at the end of exponential growth phase.

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KEYWORDS

ergosterol, *Kluyveromyces marxianus*, specific ergosterol content, shear force, aeration, two-stage fermentation

1. INTRODUCTION

Kluyveromyces marxianus is a so called non-conventional yeast belonging to the hemiascomycetes and is most commonly found in dairy products. In contrast to *Saccharomyces cerevisiae*, *K. marxianus* is capable of assimilating a variety of sugars such as lactose, which is found in whey, as well as xylose and arabinose, which are contained in lignocellulosic biomass hydrolysates (Liu et al., 2022). For this reason, this yeast has been widely used for the production of biomolecules of economic and biotechnological interests, e.g. using enzymes such as β-galactosidase, inulinase and pectinase as well as recombinant proteins, aroma compounds, and ethanol. In addition to the fermentation of lactose, *K. marxianus* has other desirable attributes for industrial fermentation processes such as thermotolerance, high growth rate, and the capacity to metabolise pentoses, hexoses, and disaccharides (Karim et al., 2020).

Ergosterol, a sterol compound found in the cell membranes of fungi, particularly in yeast and moulds, plays a crucial role in providing stability and fluidity to the lipid bilayer (Johnston et al., 2020; Jordá and Puig, 2020). This compound serves as a precursor for the synthesis of important molecules, including vitamin D_2, and possesses significant physiological functions in fungal systems (Hu et al., 2017).

The role of ergosterol in yeasts during stress conditions is a critical aspect of cellular adaptation and survival. As a key component of the fungal cell membrane, it plays an important role in the yeast's response to various stressors (Sokolov et al., 2022). Yeast cells encounter various environmental stresses, such as heat, osmotic pressure, and oxidative stress (Bhattacharya et al., 2018). Although the role of ergosterol is well-known in *S. cerevisiae*, it is a less-explored area in the case of *K. marxianus* (Blaga et al., 2018).

Understanding the dynamic role of ergosterol in *K. marxianus* under stress conditions provides insights into the cellular strategies employed by these microorganisms for survival. The interplay between ergosterol and stress response pathways in *K. marxianus* is a complex and evolving field, with implications for both basic fungal biology and potential applications in biotechnology and medicine. For the workhorse *S. cerevisiae*, the regulation mechanism is intensively researched and described, but there are still gaps. Jordá and Puig (2020) described the adaptive response of *S. cerevisiae* to sterol depletion, low oxygen, hyperosmotic stress, and iron deficiency, but none of these has been studied for *K. marxianus*. However, changes in metabolic regulation as response to ethanol stress is already described also for *K. marxianus* (Diniz et al., 2017).

2. MATERIALS AND METHODS

2.1. Microorganism

K. marxianus strain Y.00243 originated from the National Collection of Agricultural and Industrial Microorganisms (NCAIM) in Budapest, Hungary.

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2.2. Inoculum preparation

Inoculum was prepared in 250-mL Erlenmeyer flasks containing 125 mL of the inoculum medium (consisting of 10 g L^{-1} glucose, 3 g L^{-1} yeast extract, and 10 g L^{-1} peptone in distilled water). The incubation was carried out whilst constantly being shaken at 250 r.p.m. at 30 °C for 72 h in a New Brunswick Scientific incubator shaker, model Innova 40R (Connecticut, USA). The required 5% inoculum was transferred aseptically into the fermentation broth.

2.3. Complex medium

Experiments were carried out in complex fermentation broth composed of the following: 20 g L^{-1} lactose, 20 g L^{-1} yeast extract, 7 g L^{-1} $NaNO_3$, 6 g L^{-1} K_2HPO_4, 3 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 10 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, and 1 mg L^{-1} $CuSO_4 \cdot 5H_2O$ (Shang et al., 2006). Lactose was sterilised separately.

2.4. Software

Statistica 13.1 software (StatSoft, Inc., Tulsa, USA) was used for the statistical and graphical evaluation of the experimental results.

2.5. Determination of ergosterol

The fermentation broth was centrifuged at 4,000 r.p.m. for 15 min by an Hermle Z200A centrifuge. Sediments of 15 mL fermentation broth were placed into 1.5-mL Eppendorf tubes and diluted with 750 µL of 8% NaOH solution. After homogenisation, it was maintained at 85 °C for 2 h. After such cell disruption, the solution was centrifuged at 13,000 r.p.m. for 5 min. Ergosterol was extracted from the sediment consecutively three times using 96% ethanol as follows. The sediment was suspended in 750 µL ethanol, homogenised for 5 min, and then centrifuged at 13,000 r.p.m. for 5 min. The supernatant was collected in another tube. This procedure was repeated twice, each time with 500 µL of ethanol, and finally the 3 supernatants were combined resulting in $0.75 + 0.5 + 0.5 = 1.75$ mL of ethanolic solution altogether (Fisher, 1958). The collected ethanolic supernatant was quantified for ergosterol with HPLC (High-Performance Liquid Chromatography) (Shimadzu, Japan) measurements with a Kinetex XB-C18 column operated at room temperature eluted by acetonitrile as the mobile phase at a flow rate of 1 mL min^{-1}. For standard solutions, HPLC grade ergosterol (Sigma-Aldrich) was used. Samples were diluted tenfold and 10 µL was injected. Ergosterol content was detected by a UV detector at wavelength of 280 nm at room temperature.

Dry cell weight was measured by Sartorius MA35 Moisture Analyzer at 105 °C until mass constancy.

2.6. C- to N-source ratio experiment

A proper carbon and nitrogen source ratio (C- to N-source ratio) was sought by designing a central composite experiment, in which two factors, the quantity of lactose and peptone, were examined at three levels (Table 1, 1st-2nd-3rd columns). During the experiment, yeast extract was omitted from the composition of the complex medium, and only peptone served as the nitrogen source during fermentation. These shaken flask experiments were conducted at an initial pH of 5.5, a temperature of 25 °C, and shaking at 250 r.p.m. Cell growth was monitored

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Table 1. Experimental design to examine the C- to N-source ratio in *K. marxianus* fermentations

Standard run	Lactose (g L^{-1})	Peptone (g L^{-1})	Specific ergosterol content (mg g^{-1})	Dry matter content (g L^{-1})
7	20.00	5.86	3.67	6.96
5	5.86	20.00	3.72	5.69
8	20.00	34.14	3.35	9.22
3	30.00	10.00	3.32	5.78
6	34.14	20.00	3.43	8.34
2	10.00	30.00	3.9	7.55
9 (C)	20.00	20.00	0.59	9.73
1	10.00	10.00	3.14	5.8
4	30.00	30.00	3.8	8.81
10 (C)	20.00	20.00	0.64	9.82

Standard run: see subchapter 2.6.; C: Centrum point.

over four days through OD measurements. At the end of the fermentations, dry matter and ergosterol content (mg g^{-1}) were determined, which served as the outputs of the experiments along with the ergosterol concentration in the extract (mg mL^{-1}) and the calculated specific ergosterol content (mg g^{-1}) of the biomass dry weight. The analysis of variance (ANOVA) was used to obtain preliminary information about the effect of the factors.

2.7. The effect of aeration to ergosterol content in *K. marxianus*

The overall volumetric mass transfer coefficient (KLa) was measured in three different cultivation systems under two different settings in each. The experimental configurations and the measured KLa values are presented in Table 2. Of the four flasks, two were agitated by a rocking table (IKA 3D rocker) and two were mixed in a shaker (see above), with measurements taken from 250 mL flasks containing 50 mL or 150 mL of liquid for both machines. It is evident that better oxygen transfer is associated with the lower working volume in shaken flasks. For the two 300 mL bioreactors (Biostat Q, B. Braun Biotech International), there was a difference in the aeration level (as indicated in the table), and both operated with 150 mL of liquid. KLa values were determined by sulphite oxidation (static) method (Suresh et al., 2009).

These cultivations took place under the same conditions, i.e. in a complex medium with a lactose concentration of 20 g L^{-1}, at 25 °C, and an initial pH of 5.5., thus results are only

Table 2. Experimental settings and KLa values to examine the effect of aeration on specific ergosterol content of *K. marxianus*

Cultivation system (aeration)	Working/Total volume (mL)	Rotation per min (r.p.m.)	KLa (h^{-1})
Rocking table	50/250	30	1,507.5
Rocking table	150/250	30	109.4
Shaker	50/250	250	3,602.7
Shaker	150/250	250	786.2
Bioreactor (1 L min^{-1})	150/300	300	2,620.6
Bioreactor (0.1 L min^{-1})	150/300	300	1,053.5

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depending on KLa. Furthermore, the experiments were carried out simultaneously, using 5% inoculum based on the method described above. Cell growth was monitored through OD measurements, and at the end of the fermentation, dry matter and ergosterol contents were determined. Since the fermentations were conducted under identical settings, this allowed to correlate the obtained results with the KLa.

2.8. The effect of shear force to ergosterol content in *K. marxianus*

During the experiment, the impact of shear stress on the growth of yeast cells and their ergosterol content was examined in two bioreactor fermentations. Two different agitation speeds and aeration rates were determined for which the KLa values (measured by dynamic KLa determination method) (Suresh et al., 2009) were identical in both cases. In this case, the difference between the results of the two fermentations was presumably caused by shear forces, as the effect of aeration was ruled out with the same KLa value. The determined stirring speed and aeration rate combinations in the Biostat M (B Braun Biotech International) 2 L bioreactor with almost the same KLa values (56.93 h^{-1} and 56.03 h^{-1}) were 750 r.p.m. with 0.16 L min^{-1} and 410 r.p.m. with 1.6 L min^{-1}, respectively. For both fermentations, after sterilisation, decanted whey (Soma's Trade Ltd.) was used as the medium at an initial pH of 5.5 and a temperature of 28 °C was maintained.

2.9. Two step fermentation with ethanol stress

The experiment was conducted in two 1 L Biostat Q bioreactors. For the fermentation, a complex medium was utilised containing 40 g L^{-1} of lactose in a working volume of 630 mL and inoculated with 70 mL of inoculum. The agitation was consistently set at 500 r.p.m. and aeration was maintained at 0.4 L min^{-1}. During the fermentations, in the initial phase, the focus was on cell growth (higher temperature at 25 °C and no ethanol supplementation), followed by induction of ergosterol production applying unfavourable conditions (lower temperature 20 °C, and 3 V/V% ethanol stress). The change of conditions (temperature and alcohol) was done either at the 9th hour or the 21st hour after inoculation, i.e. either before or after the exponential phase.

According to our previous report, pH had no significant effect, thus was not changed between the growing and production stages (Vidra et al., 2021).

3. RESULTS AND DISCUSSION

3.1. C- to N-source ratio experiment

First, the results were examined from the perspective of cell proliferation. The quadratic effect of lactose and both the linear and quadratic effects of peptone proved to be significant. The impact of the two factors on dry matter content is illustrated in Fig. 1/A. It can be seen that a bell-shaped curve was resulted, with the highest value occurring at the composition of 22 g L^{-1} lactose and 25 g L^{-1} peptone.

In the case of the specific ergosterol content, the quadratic effect of the two factors proved to be significant. Figure 1/B illustrates how the ergosterol content of the cells changes due to the impact of the two factors. The results are particularly interesting when considering the biomass outcomes. The effect is exactly the opposite. Therefore, where yeast can grow well, the ergosterol

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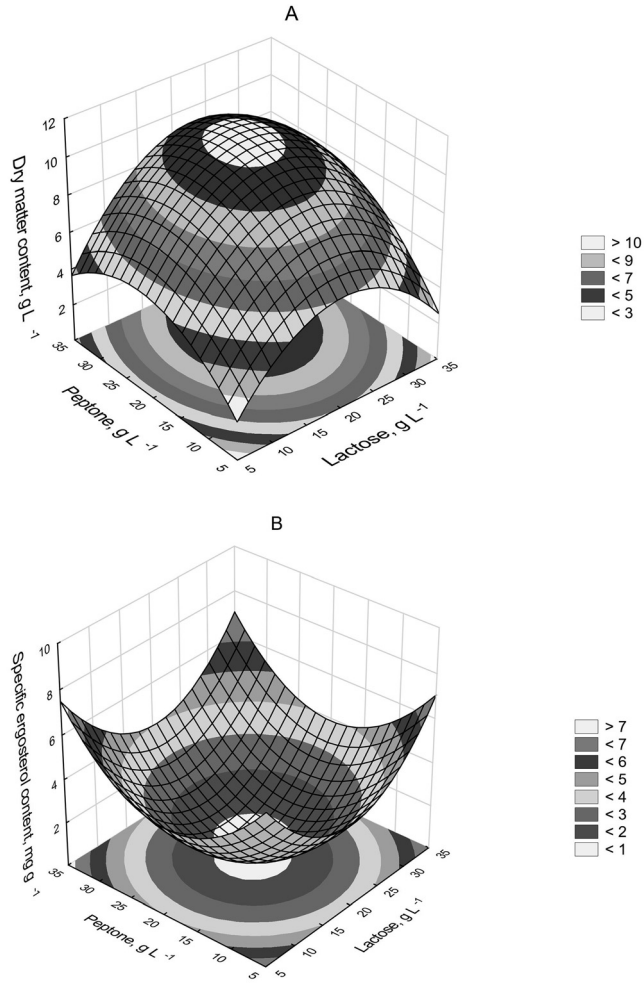

Fig. 1. Surface plots of dry matter content (left) and specific ergosterol content (right) in the C to N ratio experiment

content of the cells were very low, while at the extreme values, where fewer cells were produced, the specific ergosterol content was higher. Therefore, it is crucial in the following experiments to examine the combined effect of the two factors on the achievable ergosterol concentration (mg L^{-1}), which is the arithmetical product of biomass quantity and specific ergosterol content.

3.2. The effect of aeration to ergosterol content in *K. marxianus*

Comparing the results (Fig. 2), it can be observed that both the type of agitation and the intensity of aeration influence the amount of producible ergosterol. The specific ergosterol data clearly show that with better oxygen transfer, the ergosterol content of the cells significantly increases.

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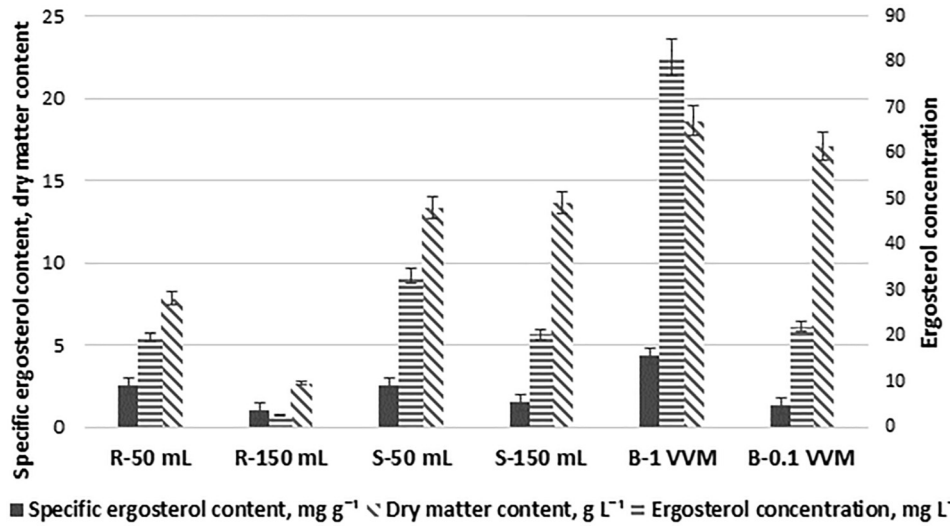

Fig. 2. The effect of aeration to ergosterol content in *K. marxianus* fermentation using different cultivation equipment (R: rocking table, S: shaker; B: bioreactor; VVM: aeration rate in volume/volume/minute)

In the case of the tilting table (3D rocker), the specific ergosterol content was 2.5 times higher (0.97, 2.51 mg g^{-1}), and for the 300 mL bioreactors, it was 3.4 times higher (1.28, 4.34 mg g^{-1}) with higher aeration settings. The dry matter content of the fermentation broth mainly exhibited significant differences in the tilting table experiment, increasing from 2.7–7.8 g L^{-1} with better oxygen transfer. Regarding the achievable ergosterol concentration, the experiment with better aeration settings proved to be more successful in all cases. Similar results were obtained by Liu and co-workers (2022), who reported that enhanced aeration increased the ergosterol titer from 63.09–128.46 mg L^{-1} using glucose as carbon source.

Based on the results, it can be stated that the level of aeration is not irrelevant for ergosterol production. Therefore, this should also be taken into account when examining the effect of shear stress.

3.3. The effect of shear force to ergosterol content in *K. marxianus*

Interesting observations could be formulated based on Fig. 3. Applying higher shear stress, the specific ergosterol content was approximately twice that of the second fermentation with lower shear force (3.9 and 1.8 mg g^{-1}, respectively). There were very few publications where the effect of stirring or shaking speed on the ergosterol content was investigated, however, in these reports the results were attributed to better aeration conditions (Ethafa and Al-Manhel, 2022). No publication was found specifically investigating the effect of agitation speed or shear force on the ergosterol content of *K. marxianus* while excluding the effect of aeration rate. In several studies, a similar approach was applied to examine *K. marxianus* β-galactosidase production and activity, resulting in finding an optimum for the combined effects of agitation and aeration rate (Schneider et al., 2001; Dagbagh and Goksungur, 2009).

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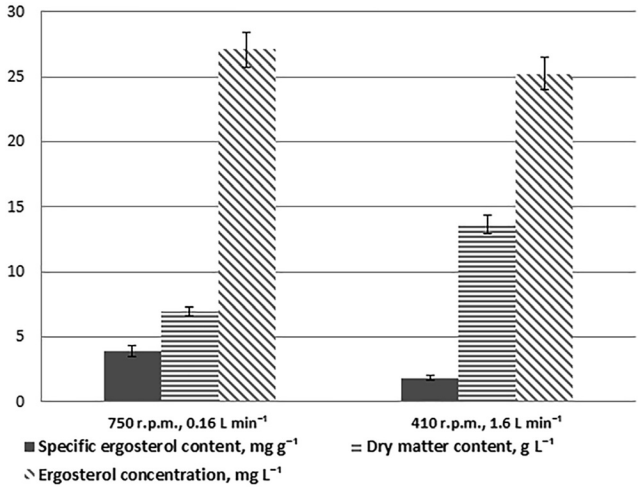

Fig. 3. The effect of shear force on ergosterol content in *K. marxianus* fermentation using different aeration rate and stirring speed while the KLa values were set to the same

The effect is opposite for dry matter content; in this case, more biomass was produced in the second fermentation with lower shear force (13.65 g L^{-1} compared to 6.93 g L^{-1}). This is understandable, as under the same aeration conditions cells grew better where they experience lower stress. As a result, the first fermentation achieved a slightly higher value in ergosterol concentration, but the two values were almost identical (27.1 and 25.3 mg L^{-1}). A notable effect was observed in dry matter content and specific ergosterol content; however, these seem to balance each other in individual fermentations.

Based on these observations together with the observed C/N response surfaces, we concluded to separate the fermentation into a growth phase and an ergosterol production phase.

3.4. Two step fermentation with ethanol stress

During the first fermentation (Fig. 4, Fermentation – A), samples were taken just before (21st h) and twice after the switch (47th h and 65th h). A significant portion of the growth had already taken place before the conditions were changed; however, growth continued afterward, likely due to the yeast starting to consume the added alcohol.

The results of dry matter and ergosterol obtained from the collected samples are illustrated in Fig. 4. Before the switch, very few cells had formed, resulting in a low ergosterol concentration, even with an average specific ergosterol content. In contrast, it was expected that with low specific ergosterol content, much more cells could be produced, since the first phase occurred under favourable conditions for cell proliferation.

After the switch, in the sample taken at the 47th h, the ergosterol content of the cells did not increase; however, the dry matter content continued to grow, almost doubling. Consequently, the ergosterol concentration also significantly increased. In the sample taken at the end of the fermentation, a much higher ergosterol content was measured, indicating that time is needed for the increase in specific ergosterol content.

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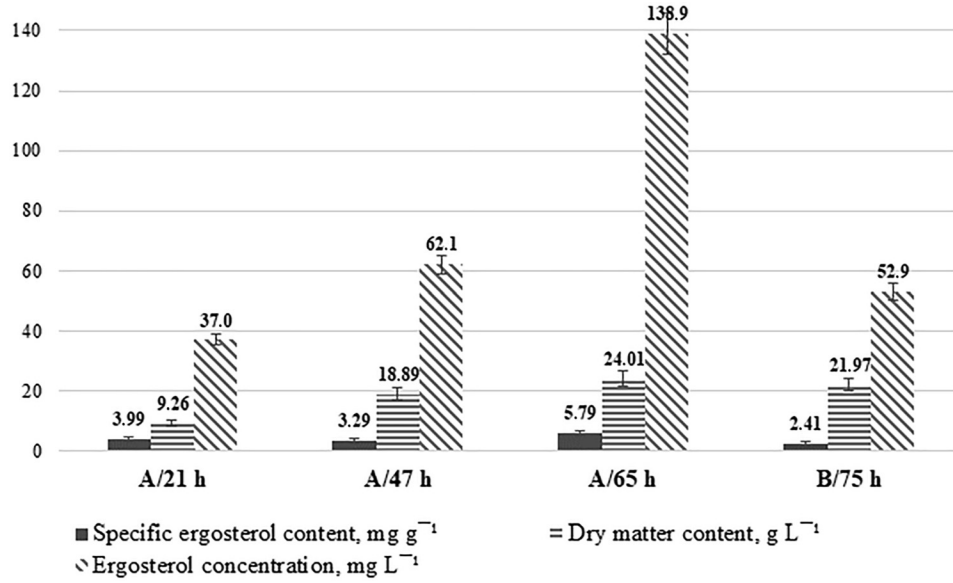

Fig. 4. The ergosterol content in *K. marxianus* two-stage fermentations applying ethanol stress at different phase of the cell growth. Fermentation A: switch after cell growth; Fermentation B: switch before exponential phase

In the second fermentation (Fermentation – B), settings were changed to the second stage at the beginning of the exponential phase (9th h). Only the sample taken at the end of the fermentation was used to measure dry matter and ergosterol values. The end results of the fermentation are presented in Fig. 4 (B/75 h). The dry matter was similarly high as in the previous experiment (Fermentation – A), despite the fermentation mostly occurred in the ergosterol production phase. Unfortunately, the ergosterol content of the cells turned out to be very low, resulting in a low ergosterol concentration.

Balbino et al. (2021) reached similar ergosterol concentrations using 2-phenylethanol as stress factor, although the achieved biomass concentration decreased from 18–9 g L^{-1} during the treatment. The duration of the stress effect was gradually increased from the beginning of the exponential phase; however, it was not investigated how the ergosterol concentration would change if it were applied later.

Based on the results, it was not worth exposing the cells to stress during the growth phase, it was enough only at the end of it, thus a larger or at least not smaller amount of biomass can be achieved with higher ergosterol content.

4. CONCLUSIONS

We observed optimal conditions for both cell growth and ergosterol content of *K. marxianus* yeast, which had no overlapping regarding experimental conditions as expected, since ergosterol serves as defending agent against stress factors. Since ergosterol synthesis highly depends on

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oxygen and plays a crucial role in defence against various stresses, effect of aeration and shear force was also investigated. These results concluded that increased shear force similarly to temperature and ethanol addition only increased the specific ergosterol content beside lower biomass yield, but at the same time, increased aeration positively affected both biomass quantity and specific ergosterol content of the cells. Thus aeration was the only parameter, which could simultaneously improve biomass and ergosterol yield. In order to achieve a higher ergosterol yield, we introduced a two-stage fermentation with separate cell growth and product formation stages, which revealed that the late application of stress conditions was beneficial, since the declining culture could induce the synthesis of ergosterol more efficiently than the shocked newborn cells. After our first successful development and report here, fine tuning of the process can be a direction for further research.

Conflict of interest: Á. Németh is a member of the Editorial Board of the journal. Therefore, they did not take part in the review process in any capacity and the submission was handled by a different member of the editorial board.

ACKNOWLEDGEMENT

Authors are grateful to Zoltán Kaleta (Progressio Engineering Buero Ltd) for supporting ergosterol determination, to MÉL Biotech K + F Kft for rocking table, and to Somasajt Kft for whey samples.

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