

Systematic Investigation of Ergosterol Fermentation by *Kluyveromyces marxianus* Y.00243 via Statistical Design

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Abstract

Ergosterol, an important pharmaceutical intermediate, is the precursor of liposoluble vitamin D₂ and cortisone. It is also a main sterol in yeast cells and responsible for structural features of membranes such as the integrity, fluidity, permeability and activity of membrane-bound enzymes. *Kluyveromyces marxianus* is able to utilize various sugars such as lactose, xylose and arabinose against *Saccharomyces cerevisiae* and is also thermotolerant. Based on these aforementioned characteristics, *K. marxianus* can be of great importance in the utilization of whey and lignocellulosic biomass. In this paper, the effect of four factors on the specific ergosterol content and yeast growth was investigated using two statistical experimental designs. The factors examined were initially added alcohol, temperature, salt concentration and pH. The initially added alcohol had a positive effect on the specific ergosterol content, resulted in 37 % specific ergosterol content increasement. The temperature had a negative effect on yeast growth reducing the biomass concentration by 50 % when increased from 25 °C to 30 °C. The pH had a significant effect only on the specific ergosterol content, having an optimum at pH 5.5. The salt concentration had no significant effect in either case. Based on the results, it is suggested that the setup which facilitates higher ergosterol content but does not slow down the growth of the yeast remarkably should be selected, which are 25 °C, pH 5.3 and 3 % of initial ethanol content.

Keywords

ergosterol, *Kluyveromyces marxianus*, experimental design, ethanol addition, optimization

1 Introduction

Kluyveromyces marxianus is a hemiascomycetous and homothallic yeast, phylogenetically close to *Kluyveromyces lactis*. 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 [1, 2]. 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 [3–8].

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 metabolize pentose, hexose and disaccharides [9, 10]. Since, for years, it used to mainly be isolated from dairy products, it possesses the GRAS (Generally

Recognized As Safe) and QPS (Qualified Presumption of Safety) statuses, therefore, making it suitable for applications in the food and pharmaceutical industries [11–13].

Ergosterol is one of the most economically important components of yeast biomass which could be used as a precursor of vitamin D₂ and other sterol drugs such as cortisone, brassinolide and progesterone [14, 15]. In yeast cells, ergosterol is found stored in its free form in plasma membrane and as esters of fatty acids [16, 17]. Similarly to the cholesterol in mammalian cells, sterol plays the main role in ensuring the integrity of cellular membranes and controlling their functions of fluidity, permeability and transport as well as the activity of proteins in plasma membranes and the cell cycle [2, 18–20]. Ergosterol not only has unique physiological functions but is also widely used in drug development [21]. Most antifungal drugs in clinical use are developed to inhibit the key enzymes of ergosterol

biosynthesis [22]. Furthermore, derivatives of ergosterol perform significant antitumor and anti-HIV activities [23, 24]. Ergosterol is also very important for adaptation to stress in fungi. It has been found that the ability of yeast to tolerate stress is closely related to ergosterol levels. For example, the ergosterol content of yeasts that are resistant to freezing and low-sugar conditions or are treated with alcohol is higher than that of common yeast [25–27].

Ergosterol is mainly produced by two different methods. Firstly, ergosterol can be extracted from waste mycelium used in the production of penicillin or during the fermentation of citric acid. Secondly, ergosterol can also be produced by the fermentation of yeast. In the former method, the necessary raw materials are much cheaper, but the ergosterol content of mycelium is lower than that of yeast [28, 29].

Since ergosterol also plays an essential role in terms of adaptation to stress during fermentation, it follows that cells growing under different conditions of environmental stress can accumulate more ergosterol. Therefore, in this study, the effects of the pH and temperature as well as ethanol addition were investigated in the first experimental design. Furthermore, the effects of the pH (over a wider range) and salt concentration were investigated in a second experimental design. With different NaCl concentration we intended to examine if this type of osmotic stress had any effect on ergosterol content of cells. Both the ergosterol content of the cells and growth of the yeast cells were examined using response surface methodology.

2 Materials and methods

2.1 Microorganism and maintenance

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

Lyophilized yeast was reactivated in 1 mL of YM medium (yeast extract: 3 g/L, malt extract 3 g/L) for 2–3 mins then cultured aseptically on solid agar plates (20 g/L malt extract and 20 g/L agar in distilled water) previously sterilized in a Tuttnauer 3870 ELV (Belgium) autoclave at 121 °C for 20 min. The inoculated solid medium was incubated at 30 °C for 4 days to facilitate the appropriate degree of growth before being stored at 5 °C for further use.

2.2 Inoculum preparation

Inoculum was prepared in 100-mL Erlenmeyer flasks containing 50 mL of the inoculum medium (consisting

of 10 g/L glucose, 3 g/L yeast extract and 10 g/L peptone in distilled water) previously autoclaved at 121 °C for 20 min. The incubation was carried out whilst constantly being shaken at 250 rpm 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 Fermentation conditions

Experiments were carried out in 250-mL Erlenmeyer flasks containing 100 mL of fermentation broth composed of the following: 20 g/L lactose, 20 g/L yeast extract, 7 g/L NaNO₃, 6 g/L K₂HPO₄, 3 g/L MgSO₄ · 7 H₂O, 1 mg/L FeCl₃ · 6 H₂O, 10 mg/L ZnSO₄ · 7 H₂O and 1 mg/L CuSO₄ · 5 H₂O [30]. The lactose content was sterilized separately. Unless otherwise stated, a temperature of 25 °C was applied. The incubation was carried out under constant shaking at 250 rpm. The prescribed ethanol addition was carried out before inoculation.

2.4 Experimental design

In order to study the effect of factors on ergosterol fermentation two central composite designs with three and two factors, respectively, were applied. All the factors were tested at three different levels. The investigated factors and their values are listed in Tables 1 and 2 for the two experimental designs, respectively.

The first experimental design was a face-centred central composite design with 3 factors at three different levels as presented in Table 1.

Table 1 Settings of the first experimental design

Standard run	Temperature (°C)	pH	Ethanol (%)
2	25	4.5	10
1	25	4.5	0
9	25	5.5	5
3	25	6.5	0
4	25	6.5	10
11	30	4.5	5
13	30	5.5	0
16 (C)	30	5.5	5
15 (C)	30	5.5	5
14	30	5.5	10
12	30	6.5	5
6	35	4.5	10
5	35	4.5	0
10	35	5.5	5
8	35	6.5	10
7	35	6.5	0

Table 2 Settings of the second experimental design

Standard run	pH	NaCl (g/L)
2	3.5	20.0
7	5.5	1.9
8	5.5	23.1
3	7.5	5.0
1	3.5	5.0
9 (C)	5.5	12.5
6	8.3	12.5
5	2.7	12.5
10 (C)	5.5	12.5
4	7.5	20.0

The second experimental design was a central composite design with two factors at three different levels as presented in Table 2.

The outputs of the experiments were the dry cell weight, the specific ergosterol content of the biomass (mg/g) and the ergosterol concentration in the extract (mg/mL). The ANalysis Of VAriance (ANOVA) was used to gain preliminary information about the effect of the factors.

The applied quadratic polynomial response model was given by this equation:

$$\hat{Y} = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^k \sum_{j=1}^k \beta_{ij} X_i \beta_{ii} X_j + \varepsilon,$$

where \hat{Y} denotes the predicted response variable, X_i stands for the independent variable, β_0 , β_i , β_{ii} and β_{ij} refer to the regression coefficients and ε represents the random error.

2.5 Software

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

2.6 Determination of ergosterol

The fermentation broth was centrifuged at 4,000 rpm 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 μ L of 8 % NaOH solution. After homogenization, it was maintained at 85 $^{\circ}$ C for 2 h. After cell disruption, the solution was centrifuged at 13,000 rpm for 5 min.

Ergosterol was extracted from the sediment consecutively three times using 96 % ethanol as follows. The sediment was suspended in 750 μ L ethanol, homogenized for 5 min and then centrifuged at 13,000 rpm for 5 min. The supernatant was collected in another tube.

This procedure was repeated twice but by applying 500 μ L of ethanol twice and the 3 supernatants were combined resulting in 1.75 mL of ethanolic solution altogether [31].

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. For standard solutions HPLC grade ergosterol (Sigma-Aldrich) was used. Samples were diluted tenfold and 10 μ L 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 $^{\circ}$ C.

3 Results and discussion

The first experimental design was used to gain information about the effect of the temperature, ethanol and pH on the ergosterol content of yeast cells and yeast growth. The effects of these three factors are shown in Fig. 1 where the vertical line denotes the threshold of significance.

The linear effect of the temperature and the effect of the interaction between temperature and ethanol were significant in terms of yeast growth. The temperature of 25 $^{\circ}$ C was favourable for cell growth. In contrast, at slightly higher temperatures, significantly lower biomass was produced. In contrast, Actař et al. [32] reported that some *K. marxianus* strains have higher metabolic activity at higher temperature. However, Costa et al. [33] studied the growth of three different strains of *K. marxianus* at different temperatures and in the presence of different alcohol concentrations. Their results similarly to ours showed

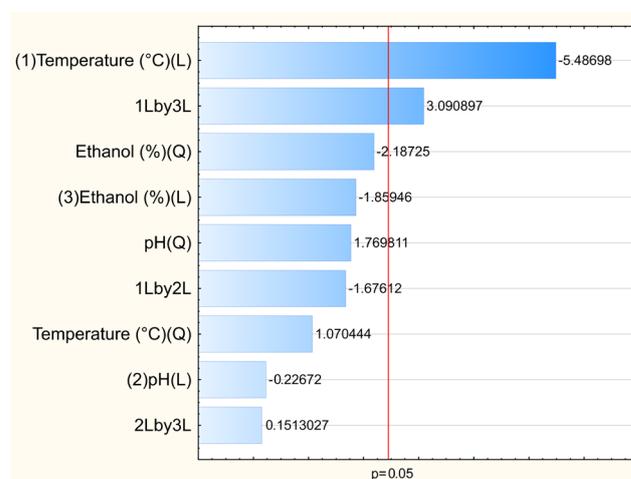


Fig. 1 Pareto chart of dry cell weight concentration in the first experimental design (L: Linear, Q: Quadratic)

that different strains tolerate higher temperatures differently i.e. our strain was less suitable for use at higher temperatures [33]. The addition of ethanol had no significant effect on cell growth, however when 10 % ethanol was introduced, lower concentration of biomass was observed. This behavior was also consistent with the results obtained by Costa et al. [33]. As is shown in Fig. 2, the higher concentration values of dry cell weight, about 11.5 g/L, were reached in the range of 0–4 % of alcohol.

The two other factors had no significant effect on cell growth within the examined range.

Evaluation of the results in terms of the specific ergosterol content of the cells concluded that the effect of the initially added alcohol and the interaction between the pH and alcohol proved to be significant as presented in Fig. 3.

In this case, the effect of the volume of the initially added ethanol has a maximum within the examined range as can be seen in Fig. 4. The highest value, 5 mg/g was reached

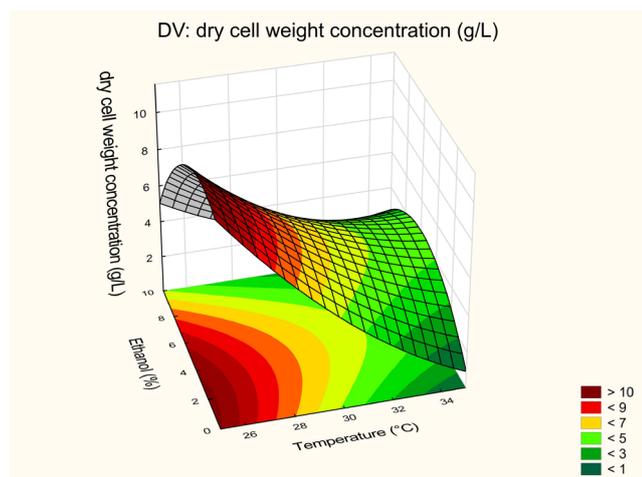


Fig. 2 The response surface plot of dry cell weight concentration as a function of ethanol and temperature

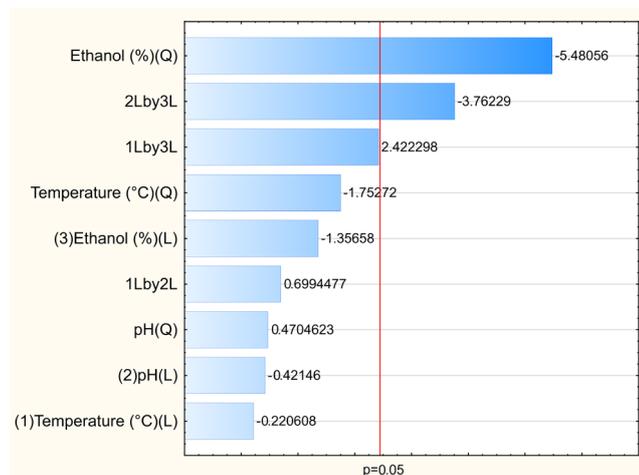


Fig. 3 Pareto chart of the specific ergosterol content of the cells in the first experimental design (L: Linear, Q: Quadratic)

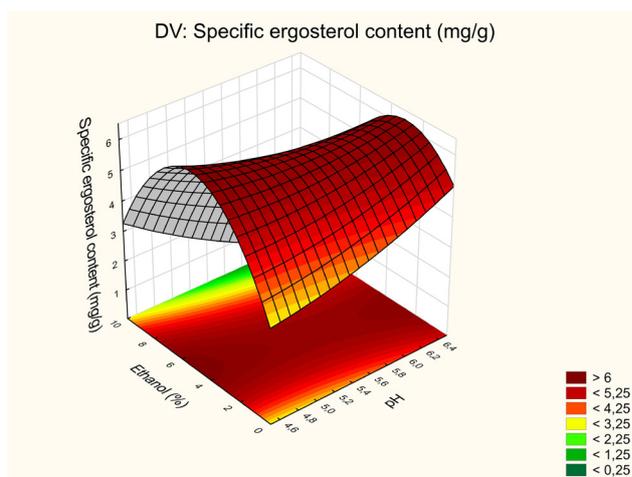


Fig. 4 Specific ergosterol content as a function of ethanol and pH

using 4.6 % ethanol. It can be seen that by increasing the amount of alcohol added, the specific ergosterol content of the cells increased. Despite the fact, Diniz et al. [1] found that ethanol exposure decreased the expression of some genes involved in ergosterol biosynthesis and as a consequent ergosterol content did not changed in *K. marxianus*, Fu et al. [34] studied the response of cells to ethanol stress at gene level and found that cells increased their ergosterol content similarly to our results. The interaction between the ethanol and pH was also visible as the saddle surface was slightly inclined.

Based on the above, it could be observed that different settings were suitable for cell growth and higher specific ergosterol content. To evaluate the overall effect of factors, a virtual ergosterol concentration was calculated from the amount of ergosterol extracted (in mg) and the volume of fermentation broth used (in L).

The initially added ethanol was again a highly significant factor with linear and quadratic effects, as can be seen in Fig. 5. The effect of the temperature was also significant. Furthermore, the interaction between the temperature and alcohol as well as the interaction between the pH and alcohol also proved to be significant.

By representing the effect of the alcohol and pH on the extractable amount of ergosterol mg ergosterol/L fermentation broth was plotted in Fig. 6. The highest value, 78 mg/L was obtained by initially adding 3 % alcohol. Interestingly, the optimal growth of *K. marxianus* at pH 5.5 is the least suitable for the production of ergosterol in terms of the virtual ergosterol concentration (the amount of extractable ergosterol in mg / volume from the volume of fermentation broth used in L). Previously, it has been observed that this microorganism did not grow well in the presence of high concentrations of alcohol [33],

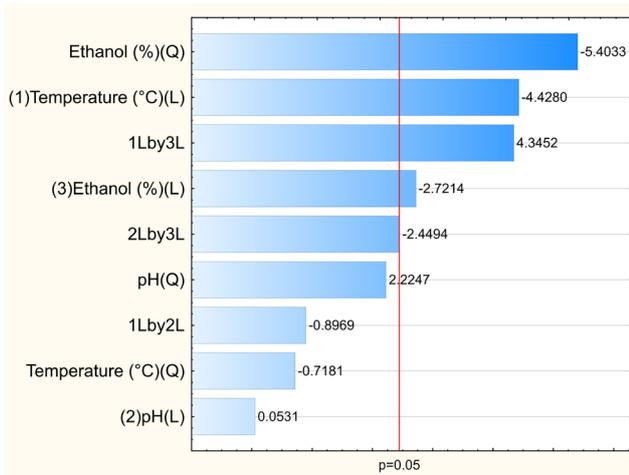


Fig. 5 Pareto chart of the virtual ergosterol concentration in the first experimental design (L: Linear, Q: Quadratic)

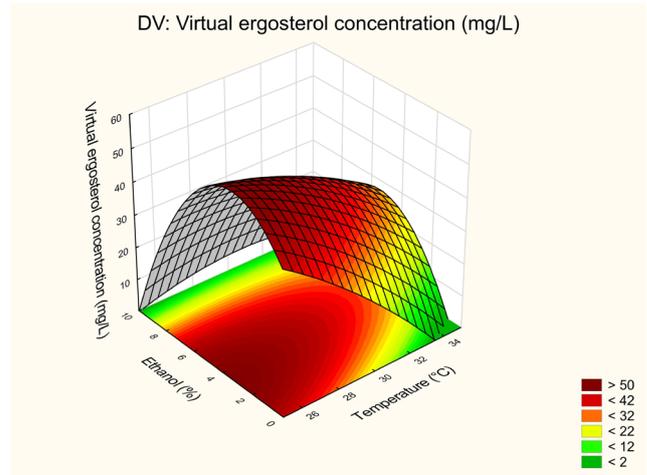


Fig. 7 Virtual ergosterol concentration as a function of ethanol and temperature

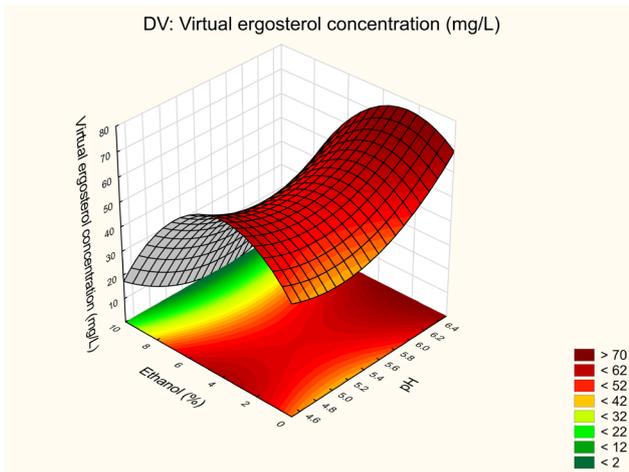


Fig. 6 Virtual ergosterol concentration as a function of ethanol and pH

which our results in Fig. 2 also confirmed: above an initial alcohol content of 8 %, the virtual ergosterol concentration results were significantly lower.

The temperature was significant in terms of the virtual ergosterol concentration which is mainly dependent on biomass growth. Here also it can be seen that the highest value, 55 mg/L was obtained by initially adding 3 % alcohol. This could affirm that it was worthwhile examining reproductive and productive conditions together. It can be seen in Fig. 7 that lower temperatures within the studied temperature range favour higher virtual ergosterol concentrations.

Since Figs. 1, 3 and 5 indicated no significant effect of pH we decided to investigate it again with wider range. Therefore, pH was tested in the next experiment along with the effect of the NaCl concentration as an additional factor.

Interestingly, neither the pH nor salt concentration had a significant effect on yeast growth within the studied range. Therefore, it is possible to adjust the pH to a value that is beneficial with regard to the production of ergosterol.

On the other hand, in terms of the ergosterol content of the cells, the effect of the pH proved to be significant quadratically as can be seen in Fig. 8.

By depicting how the specific ergosterol content of the cells changed at different pH values, a response surface was drawn and presented in Fig. 9. At the extreme values within the studied range, the minimum specific ergosterol contents were observed, while within the middle pH range, the maximum specific ergosterol contents were recorded at almost every salt concentration. The highest value was obtained at pH 5.3, which was 8.05 mg/g. Specific ergosterol content is rarely published (mostly ergosterol yield i.e. virtual concentration is given) for *K. marxianus*. The only available result was found as 0.7 mg/g [1], which is significant lower value then our result. However, industrial ergosterol fermentations generally use *S. cerevisiae*, of which reported specific ergosterol content is around

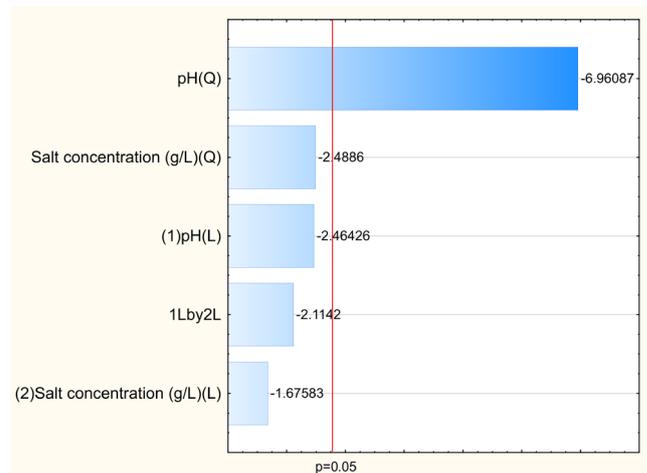


Fig. 8 Pareto chart of the ergosterol content of cells in the second experimental design (L: Linear, Q: Quadratic)

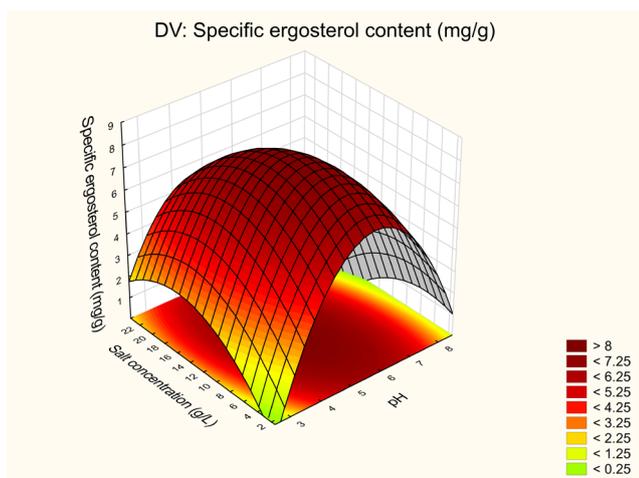


Fig. 9 Specific ergosterol content of cells as a function of the salt concentration and pH

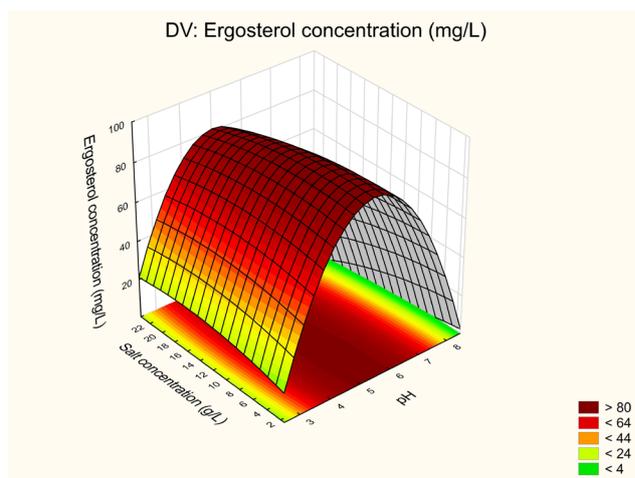


Fig. 11 Virtual ergosterol concentration as a function of salt concentration and pH

25 mg/g [30]. The fact that the salt concentration had no significant effect on the ergosterol content was confirmed since it hardly changed at different salt concentrations.

Since neither of the two factors had any significant effect on yeast growth, a similar result was expected in terms of the virtual ergosterol concentration as was observed with regard to the specific ergosterol content. Only the quadratic function of pH was found to be a significant factor in the absence of its former linear part as can be seen in Fig. 10.

The response surface was similar to the previous case (i.e. specific ergosterol content): the salt concentration did not significantly affect the virtual ergosterol concentration as is shown in Fig. 11. Our hypothesis was that cells respond to osmotic stress by increasing the ergosterol content of the cell membrane. No such research could be found in the literature. The effect of osmotic stress caused by glucose,

lactose [35, 36], and NaCl [37] on cells has been studied, however, in neither case was the amount of ergosterol or related gene regulation investigated. Lane et al. [38] showed that certain strains of *K. marxianus* were tolerant against different NaCl concentrations, however, no growth was observed in either case with 1 M NaCl solution.

In our results, the effect of NaCl concentration did not prove to be significant in the studied range, therefore, it can be concluded that this strain belongs to the group that are tolerant up to 0.5 M NaCl concentration. It may be worthwhile to investigate the effect of osmotic stress caused by different carbon sources and salts on the regulation of ergosterol content. The highest values, around 85 mg/L were reached around pH 5.3, which was the same as the pH optimum of the specific ergosterol content.

The interaction between the two factors and the effect of the salt concentration were insignificant in both cases in the second experimental design.

Verification experiments resulted in 82.3 ± 3.6 mg/L ergosterol content in three parallel runs at 25 °C and pH 5.5.

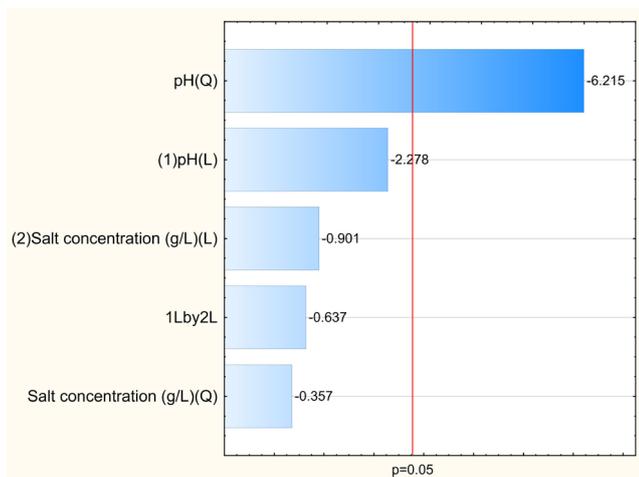


Fig. 10 Pareto chart of the virtual ergosterol concentration in the second experimental design (L: Linear, Q: Quadratic)

4 Conclusion

In summary, four factors were examined by two statistical experimental designs with regard to yeast growth and the ergosterol content of *K. marxianus* Y00243. Only temperature proved to be significant for yeast growth and had a negative effect on it. In terms of the specific ergosterol content of yeast, quadratic function of the added alcohol and the quadratic function of the pH (over a wide pH range) as well as the interaction between the alcohol and temperature proved to be significant. All these factors had a positive effect on the specific ergosterol content. Since the factors acted

differently on biomass concentration and specific ergosterol content, therefore a combined effect was evaluated through virtual ergosterol concentration (ergosterol content extracted in mg/volume of fermentation broth used in L). This evaluation proved the significance of the alcohol, temperature and pH (when a wider pH range was considered) as well as interactions between the temperature and alcohol in addition to between the pH and alcohol. The tested salt concentrations were not found to be significant in any of the tested setups.

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