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Original article

ASSESSING THE ETHANOLOGENIC POTENTIAL OF XYLOSE-FERMENTING YEASTS *SCHEFFERSOMYCES STIPITIS* UCM Y-2810

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Background. Enhancing the efficiency of second-generation (2G) bioethanol production from lignocellulosic biomass is crucial for advancing sustainable biofuel technologies. However, the conversion of biomass into 2G bioethanol faces substantial challenges, necessitating a comprehensive investigation of microbial agents.
Objective. To evaluate the effect of glucose and xylose concentrations, as well as cultivation duration, on the efficiency of ethanologenesis using the model organism *Scheffersomyces stipitis* UCM Y-2810, and to determine the optimal conditions for achieving maximum ethanol yield.
Methods. The effects of glucose and xylose concentrations and cultivation time on ethanologenesis efficiency were evaluated using *S. stipitis* UCM Y-2810 as a model organism. The experimental design included three levels of factors: xylose concentration (3, 16.5, and 30 g/l), glucose concentration (1, 5.5, and 10 g/l), and cultivation durations (1, 2, and 3 days). Statistical analysis of the experimental data was conducted using a three-factor, three-level Box–Behnken design.
Results. Under submerged cultivation of the strain of *S. stipitis* UCM Y-2810 in model media, optimization of the ethanologenesis process resulted in a maximum ethanol yield of 7.74 g/l. The optimal conditions for this yield were identified as follows: xylose concentration of 16.5 g/l, glucose concentration of 7.75 g/l, and a cultivation time of 3 days.
Conclusions. The application of the Box–Behnken design revealed that the statistically significant factors influencing ethanologenesis efficiency were xylose concentration, yeast cultivation duration, and the linear-quadratic interaction between these two factors.

Keywords: lignocellulosic biomass; xylose-fermenting yeasts; *Scheffersomyces stipites*; 2G bioethanol.

Introduction

Renewable energy plays an important role in the current and future eras to overcome and replace rapidly depleting fossil fuel reserves, reduce environmental damage by managing greenhouse gas emissions and control environmental problems associated with pollution. Fossil fuels are still the main source of energy in the world. The depletion of fossil fuels is a challenge to future availability due to the growing global population and increasing demand [1].

Most renewable energy production options are based on sources such as wind, solar, tidal, hydropower and geothermal energy, which can generate electricity and replace fossil fuels. Recently, agricultural biomass or agricultural biomass from lignocellulosic waste has gained interest due to the annual production of large volumes of agricultural biomass and the high calorific value of such biomass [2]. Lignocellulosic plant biomass (LCB), which is available in large quantities around the world, is considered an urgent feedstock for biofuel production [3]. According to estimates, LCB has the potential to generate approximately 442 billion liters of bioethanol annually. If crop residues and wasted crops are also taken into account, this figure could increase to 491 billion liters, which is approximately 16 times the current global production of bioethanol [4]. This highlights the substantial untapped potential of LCB as a renewable and sustainable feedstock for bioethanol production, offering significant opportunities to scale up biofuel production globally. Lignocellulose is the building block of plants and consists of cellulose, hemicellulose and lignin as the main components in a complex structure that prevents degradation. The production of biofuels from agricultural LCB depends on several factors, including seasonal availability, composition, strategy [5]. However, unlocking the full potential of lignocellulosic waste for bioethanol production is not without its challenges. The efficient conversion of LCB into bioethanol presents several challenges. The primary hurdle is the complex process of hydrolyzing lignocellulose into simple sugars like glucose and xylose, which

can then be fermented into ethanol [6, 7]. Traditional yeasts, such as *Saccharomyces cerevisiae*, efficiently ferment glucose but are incapable of utilizing xylose, leading to reduced ethanol yields [8]. The inclusion of xylose-fermenting microorganisms in the bioethanol production process can significantly enhance the efficiency of LCB conversion and increase ethanol yields [9]. Among yeasts, *Scheffersomyces stipitis* and *S. passalidarum* demonstrated the highest ethanol yields, exceeding 0.44 g/g, under conditions of oxygen limitation [9]. These findings highlight the potential of *S. stipitis* and *S. passalidarum* as promising candidates for efficient bioethanol production. However, to achieve optimal results, it is essential to optimize fermentation conditions, particularly the concentrations of glucose and xylose, as well as the duration of the process.

In this context, the use of yeasts capable of fermenting xylose, such as those from the species *S. stipitis*, becomes particularly significant. Incorporating this microorganism into the bioethanol production process has the potential to substantially enhance the efficiency of optimize fermentation conditions, specifically the concentrations of glucose and xylose, as well as the duration of the fermentation process. Tailoring these parameters to the specific characteristics of the yeast strain can lead to improved ethanol production and more efficient utilization of lignocellulosic feedstocks.

The objective of this study was to analyse the effect of glucose, xylose concentrations and duration of cultivation on the efficiency of ethanologenesis, using *S. stipitis* UCM Y-2810 and to determine the most optimal values for the highest ethanol yield.

Materials and Methods

Based on the results of a literature search for wild strains, our attention was drawn to the newly isolated, during the screening of strains of non-traditional yeasts capable of xylose fermentation conducted at the D.K. Zabolotny Institute of Microbiology and Virology of NASU, a representative of the genus *S. stipitis*, UCM Y-2810. This strain was isolated from rotten wood in the Holosiivskyi forest. During the initial, unoptimised analysis of of ethanogenesis efficiency, the statistical cultivation of ethanol yield was quite low, even for a high xylose concentration of 40 g/l and the absence of glucose in the medium, which according to preliminary data for representatives of the species *S. stipitis* significantly inhibits xylose fermentation for concentrations above 2 g/l [11]. Thus, the ethanol content was 2.2–2.8 g/l after the 7th day of cultivation. However, after changing the method cultivation in conical flasks on a rocking chair, with the same xylose content in the medium (40 g/l), the average ethanol yield was already 6.1 g/l at the end of the first day. at the end of the first day. It is worth noting that with increasing cultivation time, the amount of ethanol produced decreased, which the researchers attributed to re-assimilation of ethanol by yeast cells [12].

For the development of the cultivation method and conditions, we used the experience gained during previous studies of *S. stipitis* UCM Y-2810 [12, 13]. For the cultivation of yeast, we chose the method of deep cultivation in conical flasks (250 ml) on a rocking chair at a temperature of 25 °C, the stirring speed was set to 120 rpm, and the cultivation time was up to 3 days.

To prepare the inoculum, which was a yeast suspension, the culture was grown on wort agar at 25 °C for 2 days. It has been adding to the experimental medium until the concentration reached 1×10^6 cells/ml. Xylose and glucose were added according to the design of each individual run.

The liquid modified YPD liquid modified medium of the following composition:

yeast extract – 10 g/l,
peptone – 5 g/l,
xylose – from 1 to 10 g/l,
glucose – from 3 to 30 g/l.

The volume of the medium in the flask was 80 ml.

The use of mathematical statistics methods to determine of significant factors and optimal values of significant factors was used for a more in-depth study of the process of ethanologenesis of the yeast *S. stipitis* UCM Y-2810 during cultivation on mixtures of xylose and glucose. We used three-factor three-level experimental design according to Box–Behnken design (BBD) in combination with the desirability function (Design of Experiments (DOE) module in Statistica 14.01, TIBCO Software Inc.) [14]. The BBD was selected according to the actual concentrations of these monosaccharides in syrups. According to the BBD, 15 variants of the experiment were performed, each of which meant a certain combination of optimization factors, where the concentration of ethanol in the medium served as an optimization parameter [15]. The independent variables were the following factors: X_1 – xylose concentration in the medium, g/l; X_2 – glucose concentration in the medium, g/l; X_3 – duration of yeast cultivation, days (Table 1). This ap-

proach allows us to estimate linear and quadratic effects of the influence of factors (X_1, X_2, X_3) on the indicator Y and express them in the form of a regression equation within one model:

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j>i}^{n} b_{ij} x_i x_j,$$

where $b^\wedge$ is the constant, b_i is the linear coefficient, b_{ii} is the quadratic coefficient, and b_{ij} is the second order interaction coefficient.

Determination of the amount of ethyl alcohol synthesized by yeast cultures was carried out by gas was determined by gas chromatography-mass spectrometry (Laboratory of Biological Polymeric Compounds, D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine) using an Agilent 6890N/5973 inert instrument (Agilent Technologies, USA), HP-INNOWax capillary column (30 m × 0.25 mm × 0.25 μm) (J&W Scientific, USA). The separation was performed with a temperature gradient of 20 °C/min from 40 to 120 °C, helium as a carrier gas, and a flow rate of 1 ml/min through the column.

General statistical processing of the data was carried out using using Statistica 14.01 (TIBCO Software Inc., 2022). The obtained results were considered statistically significant at $p \le 0.05$.

Results

The results were obtained after 15 separate runs of the cultivation conditions which were determined by the BBD experiment design matrix are presented in Table 2.

The significance levels of the effects – linear, quadratic, and interaction – were determined through analysis of variance (ANOVA). This analysis revealed that each of the optimization factors studied, namely xylose concentration (X_1), glucose concentration (X_2), and yeast cultivation duration (X_3), were considered statistically significant ($p \le 0.05$) on both the concentration of viable yeast cells and the ethanol concentration (Table 3). These findings underscore the critical role that each factor plays in optimizing the fermentation process, as even slight variations in these parameters can markedly influence the overall yield and efficiency of ethanol production.

Table 1: Optimization factors and their values used in Box–Behnken design

Factor designation	Factor	Minimum value "–"	Average value "0"	Maximum value "+"
X_1	Xylose, g/l	3	16.5	30
X_2	Glucose, g/l	1	5.5	10
X_3	Duration, day	1	2	3

Table 2: Ethanol content after cultivation of *S. stipitis* in conical flasks according to the Box–Behnken design

Sample	Starting parameters			Results	
	X_1	X_2	X_3	Y_1	Y_2
1	3	1	2	9.279	0
2	30	1	2	9.580	2.799
3	3	10	2	9.279	0
4	30	10	2	9.279	3.375
5	3	5.5	1	9.491	2.716
6	30	5.5	1	8.491	2.305
7	3	5.5	3	9.699	2.963
8	30	5.5	3	9.398	3.787
9	16.5	1	1	8.447	0
10	16.5	10	1	8.643	0
11	16.5	1	3	9.398	5.350
12	16.5	10	3	9.643	7.738
13	16.5	5.5	2	9.643	4.198
14	16.5	5.5	2	9.908	3.128
15	16.5	5.5	2	9.519	3.210

Notes. X_1 – xylose concentration in the medium, g/l; X_2 – glucose concentration in the medium, g/l; X_3 – duration of yeast cultivation, days; Y_1 – yeast biomass, lg(CFU/ml); Y_2 – ethanol concentration, g/l.

Standardized effects of ANOVA are located according to absolute value was illustrated in Fig. 1. This diagram shows that the linear effect of the cultivation duration has the highest reliable influence on yeast biomass. The data obtained indicate that the concentration of significant ($p \le 0.05$) effect on ethanol yield had xylose concentration (linear effect, $F = 22.96$, $p = 0.040$), duration of yeast cultivation (linear effect, $F = 38.6$, $p = 0.024$) and the linear-quadratic interaction of these two factors ($F = 45.48$, $p = 0.021$), all other factors or their interactions do not significantly influence the efficiency of ethanologenesis.

Glucose is not a statistically significant factor, neither in its quadratic nor linear form, which confirms the observation made in the results of the experiment included in the experiment matrix. It is also worth mentioning the interaction effect between linear xylose and quadratic glucose, which is close to the point of statistical significance. Interestingly, this effect has a negative value, which indicates a decrease in the efficiency of ethanologenesis with a large amount of both monosaccharides in the medium – this observation aligns with the well-known phenomenon of glucose repression, but evaluating the p-value ($0.076 > 0.05$) for it, it is not is not statistically significant enough.

In the course of analyzing the raw data, a regression equation is obtained which has the form of a second order quadratic polynomial taking into account only statistically significant effects:

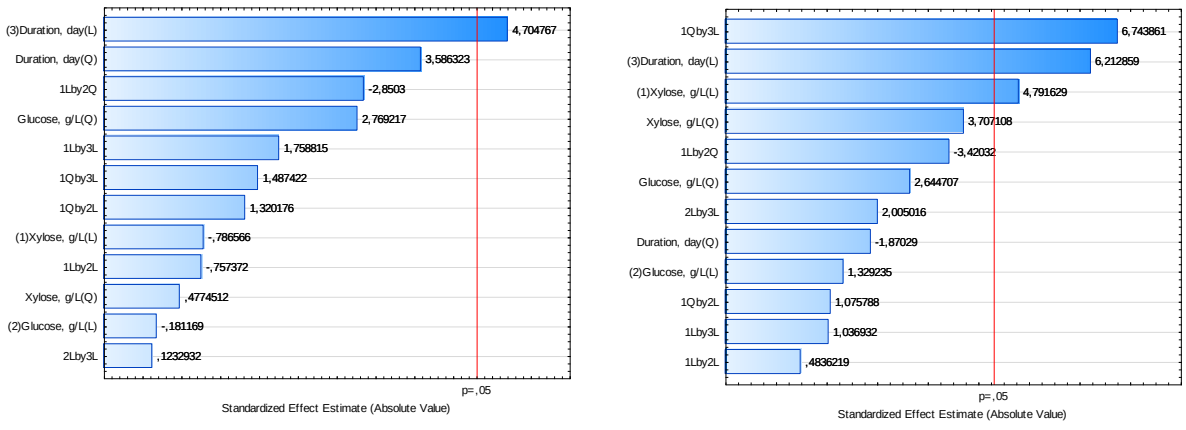
$$Y_1 = 9.218 + 0.686X_3,$$

$$Y_2 = 2.586 + 2.126X_1 + 2.757X_3 + 2.839X_1^2X_3.$$

Table 3: Results of analysis of variance (ANOVA) of the dependence of ethanol yield on the factors

Factor	Yeast biomass (Y_1), LgCFU/ml; R-sqr = 0.971; MS Residual = 0.039		Ethanol concentration (Y_2), g/l; R-sqr = 0.989; MS Residual = 0.354	
	Fisher's criterion	p-level	Fisher's criterion	p-level
(1)Xylose, g/L(L)	0.618	0.513	22.959	0.040
Xylose, g/L(Q)	0.227	0.680	13.742	0.065
(2)Glucose, g/L(L)	0.032	0.872	1.766	0.315
Glucose, g/L(Q)	7.668	0.109	6.994	0.118
(3)Duration, day(L)	22.134	0.042	38.599	0.024
Duration, day(Q)	12.861	0.069	3.497	0.202
1L by 2L	0.573	0.527	0.233	0.676
1L by 2Q	8.124	0.104	11.698	0.075
1Q by 2L	1.742	0.317	1.157	0.394
1L by 3L	3.093	0.220	1.075	0.408
1Q by 3L	2.212	0.275	45.479	0.021
2L by 3L	0.015	0.913	4.020	0.182

Notes. L – linear effects of factor; Q – nonlinear (quadratic) effects of factor.



a b

Figure 1: Factors influence on: (a) yeast biomass, (b) ethanol concentration

Response surface analysis can provide a better understanding of the dependencies and of factors, both dependent and independent. For yeast biomass, the most significant effect was observed in relation to the duration of cultivation, with a noticeable increase in cell numbers as the cultivation period extended (Fig. 2).

Fig. 3 shows the response surfaces of the time dependence on xylose concentration for different glucose concentrations determined by BBD of 3-factor analysis. Comparing the 3 graphs, we observe insignificant influence of glucose concentration, which once again proves the lack of statistical significance of this factor. We can also observe a certain optimum for the xylose content (15–18 g/l), which changes slightly for different glucose concentrations.

Evaluating the response surfaces for time versus glucose concentration at varying xylose concentrations (Fig. 4) reveals the role of cultivation time in determining ethanol yield. At low xylose concentrations, ethanol yield remains very low (<3 g/l)

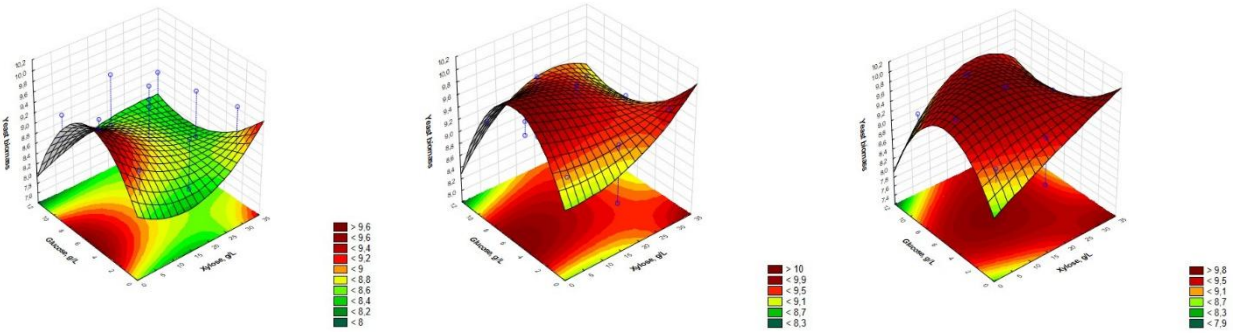


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Figure 2: Effect of xylose concentration on yeast biomass at different cultivation times: (a) 1 day, (b) 2 days, (c) 3 days

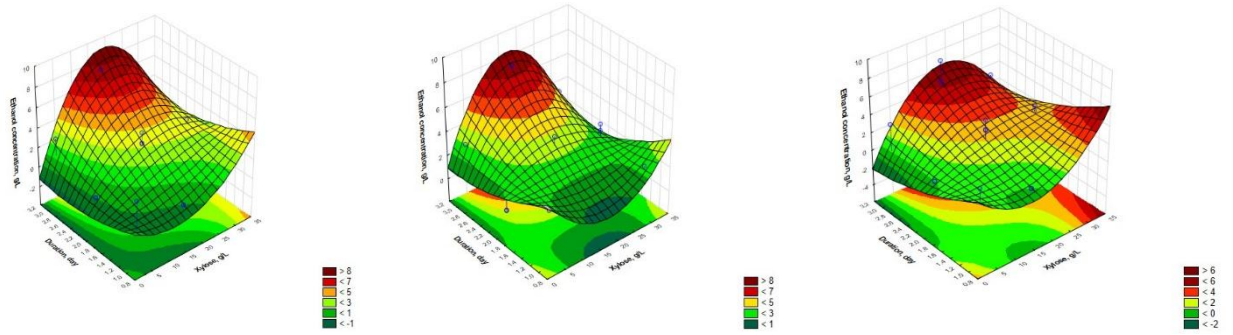

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Figure 3: The response surface of ethanol yield at glucose concentration: (a) 1 g/l, (b) 5.5 g/l, (c) 10 g/l

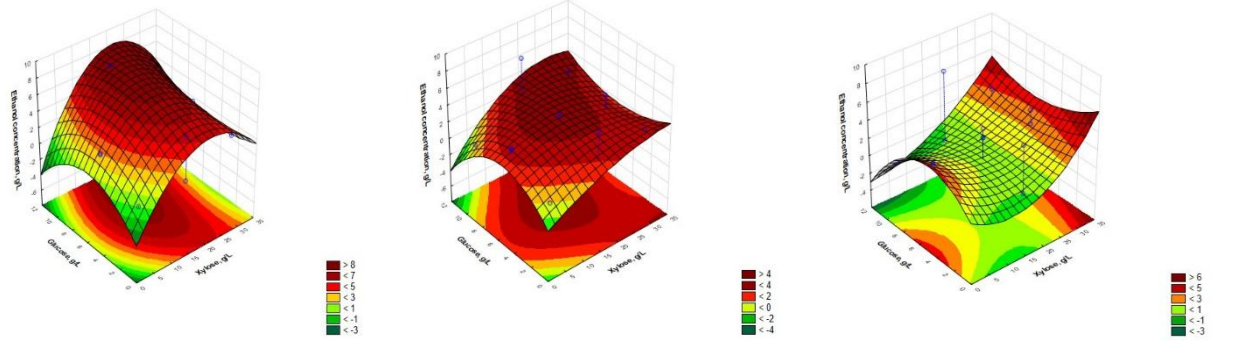

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Figure 4: Response surface of ethanol yield as a function of glucose and xylose concentrations at different cultivation times: (a) 1 day, (b) 2 days, (c) 3 days

and does not significantly change with prolonged cultivation, although a distinct glucose optimum is observed in the range of 4–6 g/l. From Fig. 4b, it can be concluded that glucose concentration has a minimal impact on ethanol yield, suggesting that other factors, such as xylose concentration and cultivation time, play more significant roles. Fig. 4c further illustrates that achieving higher ethanol yields requires an optimal xylose concentration of 16.5 g/l, combined with higher glucose concentrations and extended cultivation periods.

To determine the optimal conditions, we employed a desirability function, which integrated all the data obtained from the response surfaces. This approach allowed us to identify the optimal cultivation conditions for maximizing bioethanol yield. The desirability function works by transforming all responses into a dimensionless value (Fig. 5).

Fig. 5 shows that the most optimal cultivation time is from 3 days. If we determine the the optimal concentrations of xylose and glucose as a function of time, the general range should include xylose concentrations between 12 and 24 g/l and glucose concentrations starting from 4 g/l. However, upon analyzing the optimization graph for the balance of glucose relative to xylose, the practical optimum narrows to a xylose concentration of 17–21 g/l and a glucose concentration of 8–11 g/l.

By evaluating the profiles of predicted values (Fig. 6), the optimal conditions for maximum etha-

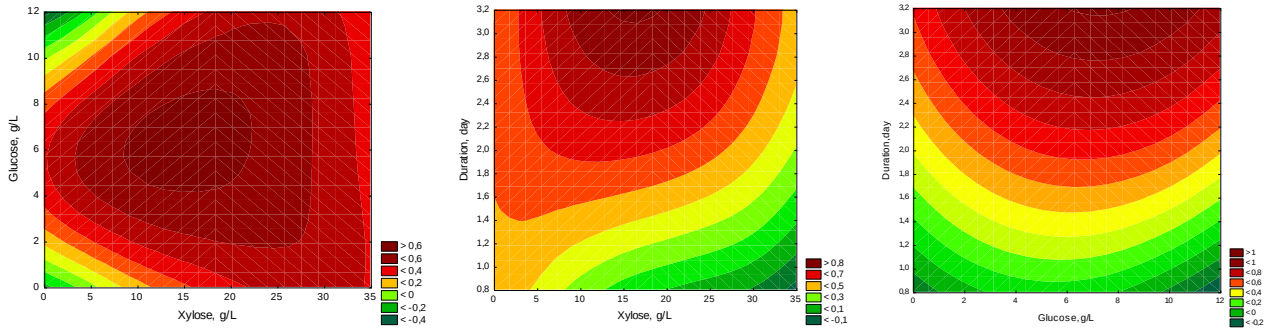

Figure 5: Dependence of the desirability function of ethanol production on the (a) xylose concentration, (b) glucose concentration, (c) duration of yeast cultivation

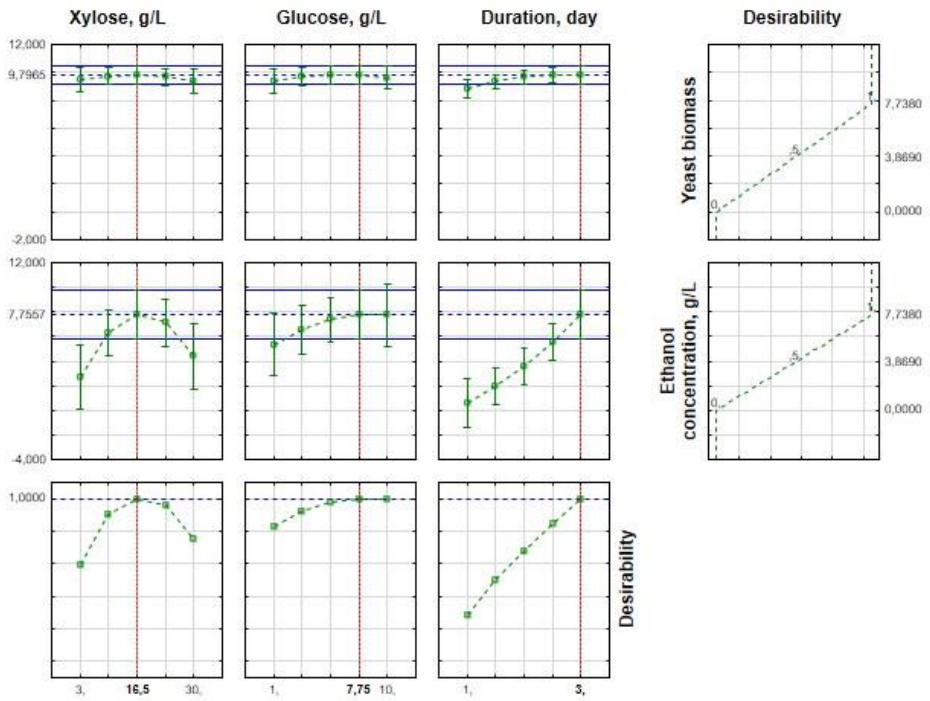

Figure 6: Profiles of predicted values for optimal ethanol concentration for Box–Behnken experimental designs using conical flasks

nol yield were determined as follows: xylose concentration of 16.5 g/l, glucose concentration of 7.75 g/l and a cultivation time of 3 days. Under these conditions, the maximum ethanol concentration achieved in the rocking flasks was 7.738 g/l.

Weak effect of glucose concentration on ethanologenesis observed at the onset of cultivation significantly diminished as the cultivation period extended. This trend may suggest a rapid initial assimilation of glucose by *S. stipitis* UCM Y-2810, compared to xylose [16]. However, it was noted that the lowest xylose concentration at the conclusion of the cultivation period led to a decrease in ethanol concentration. This observation underscores the importance of maintaining sufficient xylose levels throughout the process to sustain high ethanol yields.

Discussion

This study examines the potential for processing lignocellulosic agricultural waste into second-generation ethanol using xylose-fermenting yeast. This is highly significant for investigating ways to enhance the sustainability of bioethanol production from LCB as an alternative fuel source. It contributes to addressing issues related to sustainable development and waste management. The conditions for ethanol production were based on data regarding the content of monosaccharides such as glucose and xylose, including their concentrations and ratios. We employed statistical methods, specifically design of experiments, to optimize the fermentation conditions.

It is well known from the literature that the selection of xylose concentration in the medium, as well as the ratio of glucose to xylose in hexose and pentose mixtures, plays a crucial role in studying the ethanol-producing potential of xylose-fermenting yeast [16]. It is known that among yeasts, the highest level of xylose-fermenting activity has been observed in representatives of the species *S. stipitis* [17]. For representatives of the species *S. stipitis*, a key prerequisite for effective fermentation of cellulose hydrolysate to ethanol is the ability to ferment both glucose and xylose [11]. The investigation of the physiological characteristics of yeast consumption of glucose and xylose in ethanol production was oriented towards data obtained from yeast cultivation in most previous studies [12, 13]. It has been shown that isolates *S. stipitis* possessed the ability to ferment xylose at elevated temperatures and produced up to 6 g/l ethanol from 40 g/l xylose [12]. Additionally, in our experiments, we utilized monosaccharide concentrations and a xylose-to-glucose ratio of 3:1 in the mixtures, based on our previous data [13].

Even a weak effect of glucose concentration on ethanologenesis at the very beginning of cultivation significantly decreased with the duration of cultivation, which may indicate a rapid initial assimilation of glucose by *S. stipitis* UCM Y-2810 in comparison to xylose [16]. The influence of xylose on ethanologenesis was characterized by a positive correlation between ethanol production and both xylose concentration and cultivation time. However, the lowest xylose concentration at the end of cultivation resulted in a decrease in ethanol concentration. At xylose concentrations within the mid to high range, glucose concentration had minimal effect on the fermentation process.

To make the bioconversion of LCB an economically viable option, innovative solutions to technological challenges related to the processing and pretreatment of raw materials are essential.

Conclusions

When using the Box–Behnken design, the statistically significant factors for the efficiency of ethanologenesis were the xylose concentration, the duration of yeast cultivation, and the linear-quadratic interaction of these two factors.

The amount of the highest ethanol yield was 7.738 g/l, with the optimal factors being: xylose concentration – 16.5 g/l, glucose concentration – 7.75 g/l and time – 3 days.

These findings highlight the critical role of substrate availability and fermentation time in optimizing ethanol production efficiency. The study revealed that the tested glucose concentrations did not have a significant impact on the process of xylose fermentation by yeast. Furthermore, no evidence of non-competitive inhibition of xylose transport by glucose into yeast cells was observed, indicating that glucose did not interfere with xylose uptake or metabolism under the studied conditions.

Interests disclosure

The authors have no relevant financial or non-financial interests to disclosure. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European. Neither the European Union nor the granting authority can be held responsible for them.

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ОЦІНКА ЕТАНОЛОГЕННОГО ПОТЕНЦІАЛУ КСИЛОЗОФЕРМЕНТУЮЧИХ ДРІЖДЖІВ *SCHEFFERSOMYCES STIPITIS* UCM Y-2810

Проблематика. Підвищення ефективності виробництва біоетанолу другого покоління (2G) з лігноцелюлозної біомаси має вирішальне значення для розвитку сталих біопаливних технологій. Однак перетворення біомаси на біоетанол 2G стикається зі значними труднощами, що зумовлює необхідність всебічного дослідження мікробних агентів.
Мета. Оцінити вплив концентрації глюкози, ксилози та тривалості культивування на ефективність етанологенезу при використанні *Scheffersomyces stipitis* UCM Y-2810, а також визначити оптимальні умови для досягнення максимального виходу етанолу.
Методика реалізації. Оцінку впливу концентрації глюкози, ксилози та тривалості культивування на ефективність етанологенезу проводили з використанням *S. stipitis* UCM Y-2810 як модельного організму. Експериментальний дизайн включав три рівні факторів:

концентрація ксилози (3, 16,5 і 30 г/л), концентрація глюкози (1, 5,5 і 10 г/л) і тривалість культивування (1, 2 і 3 дні). Статистичний аналіз отриманих результатів здійснювали з використанням трифакторного, трирівневого експериментального дизайну Бокса–Бенкена.

Результати. При глибинному культивуванні штаму *S. stipitis* UCM Y-2810 на модельних середовищах оптимізація процесу етанологенезу дала змогу досягти максимального виходу етанолу 7,74 г/л. Оптимальними умови для досягнення цього виходу були: концентрація ксилози 16,5 г/л, концентрація глюкози 7,75 г/л і тривалість культивування 3 доби.

Висновки. Застосування дизайну Бокса–Бенкена показало, що статистично значущими факторами, які впливають на ефективність етанологенезу, є концентрація ксилози, тривалість культивування дріжджів та лінійно-квадратична взаємодія між цими двома факторами.

Ключові слова: лігноцелюлозна біомаса; ксилозоферментуючі дріжджі; *Scheffersomyces stipites*; біоетанол 2G.