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THE 2-PHENYLETHANOL AND ETHANOL PRODUCTION BY YEAST *SACCHAROMYCES CEREVISIAE*

The present study is focused on the determination of the 2-phenylethanol and ethanol production with use of statistical analyses experiments combined with Box-Behnken design under the influence of sucrose, yeast extract and L-phenylalanine concentrations. The effect of each factor as well as their interactions was assessed by ANOVA technique. S. cerevisiae UCM Y-514 and UCM Y-524 produced the highest calculated adjusted level 1.21 g/l of 2-phenylethanol. Meanwhile, the level of ethanol reached 22.39 g/l. The regression equations derived from statistical analysis of three main effects and the models with multiple correlation coefficients were obtained.

K e y w o r d s : S. cerevisiae, 2-phenylethanol, ethanol, optimization, statistical analysis.

Alcohol fermentation is a process used for formation of many different aroma compounds. High aromatic alcohols are responsible for the taste of beer and wines. They influence the flavours and quality of champagne and muscatel wines. One of the main commercially utilized higher aromatic alcohols that are produced by alcohol fermentation is 2-phenylethanol. Due to its strongly pronounced aroma of roses, this type of alcohol is widely used in the production of a variety of alcoholic beverages throughout the industry.

Various yeast including *Saccharomyces cerevisiae* [3, 5, 6, 12–14], *S. bayanus* [14], *Kluyveromyces marxianus* [4–6], *K. lactis* [8], *Pichia fermentans* [3, 4, 6, 7], *P. anomala*, *P. membranaefaciens* [5], *Candida utilis* [11], along with many others have been reported to be capable of producing 2-phenylethanol.

The genera *Saccharomyces* is able to produce significant amounts of 2-phenylethanol. For that reason, *Saccharomyces cerevisiae* is currently receiving attention as a yeast having a great prospect in development of biotechnological production of 2-phenylethanol. Previously, several groups of researchers have conducted series of experiments in 2-phenylethanol production with effects of sucrose and L-phenylalanine on yeast. *S. cerevisiae* DSMZ 70487 has been reported to be able to produce up to 0.34 g/l of 2-phenylethanol [5]; *S. cerevisiae* NCYC has been known to produce

1.5 g/l [1]; mutant *S. cerevisiae* is capable to result in 750 mg/l of 2-phenylethanol; *S. cerevisiae* K-9 was able to produce 1.3 g/l [4]. During the experiment, different cultivations parameters, such as precursor concentrations, cultivation temperature, initial pH and others were put under consideration. Though it has not been exactly established which of the three main effects has played the main role in the 2-phenylethanol and ethanol syntheses? It is well known that *Saccharomyces* yeast is Crabtree-positive. Consequently, the yeast can produce ethanol under aerobic conditions as a toxic by-product. Thus the knowledge of the bioconversion of L-phenylalanine to 2-phenylethanol with the usage of sucrose or glucose as carbon and energy sources is rather useful [4—7]. The estimation of the significant yeast extract concentrations in the production of the 2-phenylethanol and ethanol has been carried out only to a limited extent [7, 9].

The purpose of the present study is to determine the maximum levels of the 2-phenylethanol and ethanol production by yeast *S. cerevisiae* UCM Y-514 and UCM Y-524 using surface methodology combined with Box-Behnken design under the influence of sucrose, yeast extract and L-phenylalanine concentrations.

Materials and methods

Yeast strain and inoculum preparation. The yeast strains *Saccharomyces cerevisiae* UCM Y-514 and UCM Y-524 from the collection of yeasts (UCM) of the Industrial Microorganisms Physiology Department, Institute of Microbiology and Virology of National Academy of Sciences of Ukraine, Kiev were used in this study.

The yeasts inoculums (10^6-10^7 cells/ml) were grown in a medium, containing (g/l): 80 sucrose, 7 L-phenylalanine, 22.8 $Na_2HPO_4 \cdot 2H_2O$, 10.3 citric acid, 0.5 $MgSO_4 \cdot 7H_2O$, 0.17 Bacto Yeast Nitrogen Base without amino acids and $(NH_4)_2SO_4$, adjusted to pH 5.0. To optimize experiments we used yeast strains incubated at periodic culture in a different medium, containing 150 ml in different combination according to Box-Behnkan experimental design: 0%, 9%, 18% sucrose and 0%, 0.75%, 1.5% yeast extract, 0 mg/l, 0.1 mg/l, 0.2 mg/l L-phenylalanine in 750 ml Erlenmeyer flasks for 26 h at 28—30 °C, adjusted to pH 5.0 with HCl and shaking at 240 rpm.

Biomass and medium pH determination. Growth of yeast biomass after cultivation was measured as optical density at 540 nm and recalculated as biomass dry weight. Medium pH was determined directly with pH meter (Model pH-150MA, Antex, Byelorussia). All data was standardized and carried out in triple frequency.

GC/MS analysis. The concentrations of 2-phenylethanol and ethanol in the medium were measured after filtrations by GC/MS analyses. Samples were filtered through 0.2 μm filters prior to GC/MS analyses (Agilent Technologies, USA). GC/MS analyses were performed on Agilent 6890N/5973 inert chromatograph / mass spectrometer (Agilent Technologies, USA) equipped with DB-FFAP capillary column (30 m x 0.25 mm x 0.25 μm, J&W Scientific, USA). Helium was used as the carrier gas at a flow rate of 1 ml/min. The temperature program was as follows: 60 °C for 1 min and then increased to 220 °C at a rate 20 °C/min and held for 10 min. The temperature of injector was maintained at 250 °C. Detection was followed at SCAN rate. 2-phenylethanol and ethanol were identified by NIST 02 mass spectrum database and 2-phenylethanol and ethanol standard solution (Merck, Germany).

Statistical analysis. The conditions for biomass, 2-phenylethanol and ethanol syntheses were optimized with respect to sucrose (X_1), yeast extract (X_2) and L-

phenylalanine (X_3) concentrations using the Box-Behnkan experimental design with the software STATISTICA 6.0 (StatSoft Inc., 2001). This design determines the effect of a combination of process factors (X_1, X_2, X_3), each at three equidistant levels (-1, 0, +1) and their interactions on the response variable, which were the biomass yield (Y1), 2-phenylethanol (Y2) and ethanol (Y3) syntheses. In total, 15 combinations of factors were used. The analysis of variance technique was used to determine the significant difference at 95% confidence ($p \leqslant 0.05$) level. The effect of each factor and their interactions was assessed by ANOVA technique. The desirability function to get optimal biomass yield, 2-phenylethanol and ethanol syntheses were fitted by the least square method using the software. The 3D response graph and profile for predicted values and desirability level for factors were plotted using the software.

Results and discussion

The high aromatic alcohols are responsible for the taste of beer, white and muscatel wines, and also negatively influence the taste and quality of sparkling wines [11]. Our previous work has revealed that among sixteen *S. cerevisiae* yeast strains tested, two produce the highest yield of 2-phenylethanol. *S. cerevisiae* UCM Y-514 produced 0.11 g/l; 0.12 g/l was produced by *S. cerevisiae* UCM Y-524. Both were obtained from wine making. These strains are capable of achieving high product yields in different cultivation conditions [10].

Previously, neither reports on optimization of 2-phenylethanol and ethanol syntheses by yeast *S. cerevisiae* nor 2-phenylethanol production using surface methodology combined with Box-Behnken design for biomass growth has been done. For our experiments the regression coefficients for the main effects and their interactions were obtained by the regression analysis of the optimized experimental data to fit suitable regression equations for biomass yield, 2-phenylethanol and ethanol productions as a function of linear and quadratic effects of main factors and the linear-by linear interaction effects with regression coefficients. The regression equations obtained were response variable biomass yield, 2-phenylethanol and ethanol productions for *S. cerevisiae* UCM Y-514 and UCM Y-524. The analyses regression equations showed the complicate nature of all interactions.

The regression equations obtained for *S. cerevisiae* UCM Y-514 were

$$Y(1) = 1.63 + 0.22 \cdot X_1 + 0.14 \cdot X_1^2 + 0.33 \cdot X_2 + 0.17 \cdot X_1 \cdot X_2 + 0.17 \cdot X_1^2 \cdot X_2$$

R^2: *0.99668* (1),

$$Y(2) = 0.22 - 0.26 \cdot X_1 - 0.15 \cdot X_1^2 - 0.031 \cdot X_2 + 0.08 \cdot X_2^2 - 0.09 \cdot X_3 - 0.05 \cdot X_3^2 + 0.064 \cdot X_1 \cdot X_2 - 0.12 \cdot X_1 \cdot X_2^2 + 0.03 \cdot X_1^2 \cdot X_2 + 0.18 \cdot X_1 \cdot X_3 + 0.07 \cdot X_1^2 \cdot X_3$$

R^2: *0.99997* (2),

$$Y(3) = 6.48 + 0.89 \cdot X_1 + 3.41 \cdot X_1^2 + 3.19 \cdot X_2 + 0.7 \cdot X_3 + 1.82 \cdot X_3^2 + 6.67 \cdot X_1 \cdot X_2 + 1.53 \cdot X_1 \cdot X_2^2 + 4.98 \cdot X_1^2 \cdot X_2 + 0.68 \cdot X_1 \cdot X_3 + 0.73 \cdot X_1^2 \cdot X_3$$

R^2: *0.99998* (3),

where Y(1) — biomass yield, CDW, g/l, and 1.63; 0.22 etc. regression coefficients;

Y(2) — 2-phenylethanol production, mg/ml, and 0.22; 0.26 etc. regression coefficients;

Y(3) — ethanol production, mg/ml, and 6.48; 0.89 etc. regression coefficients.

The regression equations obtained for *S. cerevisiae* UCM Y-524 were

$$Y(1) = 1.82 + 0.41 \cdot X_2 + 0.17 \cdot X_3^2$$

R^2: *0.98632* (4),

$$Y(2) = 0.29 - 0.32 \cdot X_1 - 0.18 \cdot X_1^2 - 0.13 \cdot X_2 + 0.11 \cdot X_2^2 - 0.083 \cdot X_3 + 0.21 \cdot X_1 \cdot X_2 - 0.19 \cdot X_1 \cdot X_2^2 + 0.11 \cdot X_1^2 \cdot X_2$$

R^2: *0.99948* (5),

$$Y(3) = 6.43 + 1.91 \cdot X_1^2 + 7.54 \cdot X_2 + 1.9 \cdot X_2^2 + 1.91 \cdot X_3 + 3.57 \cdot X_3^2 + 3.21 \cdot X_1 \cdot X_2^2 - 1.66 \cdot X_1^2 \cdot X_2 + 2.56 \cdot X_1^2 \cdot X_3 + 5.32 \cdot X_2 \cdot X_3$$

R^2: *0.99816* (6),

where Y(1) — biomass yield, CDW, g/l, and 1.82; 0.41 etc. regression coefficients;

Y(2) — 2-phenylethanol production, mg/ml, and 0.29; 0.32 etc. regression coefficients;

Y(3) — ethanol production, mg/ml, and 6.43; 1.91 etc. regression coefficients.

The results showed the ability of yeast to grow on any substrates, like sucrose, yeast extract or L-phenylalanine and their variations (Table 1). The regression equations data indicated that the significant factors for the first strain were sucrose and yeast extract concentrations whereas for the other were yeast extract and L-phenylalanine. In regard to 2-phenylethanol and ethanol production the significant factors were sufficiently all main factors. We can note that the yeast extract demonstrated its ability to affect 2-phenylethanol and ethanol syntheses. This could be explained by the fact that yeast extract in addition to providing adequate nitrogen contains various vitamins which increased growth of the yeast strains. The received results completely coincided with the results of other authors [1, 7, 9]. The values of the determination coefficients for biomass yield by *S. cerevisiae* UCM Y-514 and UCM Y-524 (R^2: 0.99668 and R^2: 0.98632), for 2-phenylethanol production (R^2: 0.99997 and R^2: 0.99948), for ethanol production (R^2: 0.99998 and R^2: 0.99816) indicates that only 0.33 and 1.36%, 0.003 and 0.052%, 0.002 and 0.18%, respectively, of the total variation is not explained by the model, indicating the high goodness of fit of the model.

The effect of each factor and their interactions was assessed by ANOVA technique. The analysis of variance shows that the models are very significant for *S. cerevisiae* UCM Y-514 and UCM Y-524 (Table 2). That was confirmed by Fisher F test, where F values were calculated.

The closeness of the observed and the adjusted data can be noted which indicates that all regression equations can be used to determine biomass yield, 2-phenylethanol and ethanol concentrations at different levels of factors. Correlation coefficients were close to 1.0 and an excellent correlation between the experimental and adjusted values were obtained.

Also, we received the response surface graph of the effect of sucrose and yeast extract concentrations. It indicates that the biomass, 2-phenylethanol and ethanol productions were highly influenced by the increasing yeast extract concentrations in the medium. The lowest addition of the yeast extract determined an increase in 2-phenylethanol production (flowery perception) while the high concentrations of the yeast decreased the high aroma alcohol concentrations. The last can be explained by the formation of some carboxylic acids [2]. Graphical study accorded to the high significance of the regression model.

Table 1

Combination of independent factors for optimization experiments and response variables for *S. cerevisiae* UCM Y-514 and *S. cerevisiae* UCM Y-524

Run N	Su*, % (X_1)	YE, % (X_2)	L-ph, mg/l (X_3)	B, g/l, (Y1)	2-PE, g/l, (Y2)	Eth, g/l, (Y3)	Detected compounds
***S. cerevisiae* UCM Y-514**							
1	0	0	0.1	1.25	0.49	13.61	2-PE, Eth*
2	18	0	0.1	1.37	0	0	AA, FA, F, P, PN, FC
3	0	1.5	0.1	1.35	0.26	0	2-PE, AA, FA, B, P, F
4	18	1.5	0.1	2.14	0.026	13.08	2-PE, Eth
5	0	0.75	0	1.35	1.21	0	2-PE
6	18	0.75	0	1.75	0	4.51	Eth, AA
7	0	0.75	0	1.325	1.21	0	2-PE
8	18	0.75	0.2	1.78	0.083	7.26	2-PE, Eth, AA, FA, B, P, PN, FC
9	9	0	0	1.25	0	0	AA, FA, F, P, PN, FC
10	9	1.5	0	2.48	0	18.22	Eth, AA, FA
11	9	0	0.2	1.27	0	0	AA, FA, F, P, PN, FC
12	9	1.5	0.2	2.24	0.035	21.15	2-PE, Eth, AA, FA, P
13	9	0.75	0.1	1.86	0.041	13.41	2-PE, Eth, AA, FA, P
14	9	0.75	0.1	1.78	0.060	13.32	2-PE, Eth, AA, FA, P
15	9	0.75	0.1	1.90	0.053	13.51	2-PE, Eth, AA, FA, P
***S. cerevisiae* UCM Y-524**							
1	0	0	0.1	1.75	0.84	0	2-PE
2	18	0	0.1	1.35	0.02	0	2-PE, AA, FA, F, P, FC
3	0	1.5	0.1	2.50	0.008	19.51	2-PE, Eth, AA, FA, B
4	18	1.5	0.1	2.50	0.05	15.10	2-PE, Eth
5	0	0.75	0	1.45	1.21	0	2-PE
6	18	0.75	0	2.10	0.03	10.22	Eth, AA
7	0	0.75	0	1.75	1.21	0	2-PE
8	18	0.75	0.2	2.00	0.06	11.06	2-PE, Eth, AA, FA, B, P, PN, FC
9	9	0	0	1.325	0	0	AA, FA, F, P, PN, FC
10	9	1.5	0	1.50	0.04	0	2-PE, AA, FA
11	9	0	0.2	1.30	0.008	0	2-PE, AA, FA
12	9	1.5	0.2	2.30	0.05	21.30	2-PE, Eth, AA
13	9	0.75	0.1	2.20	0.08	17.07	2-PE, Eth, AA
14	9	0.75	0.1	1.96	0.12	16.60	2-PE, Eth, AA
15	9	0.75	0.1	2.00	0.07	15.20	2-PE, Eth, AA

* Su – sucrose, YE – yeast extract, L-ph – L-phenylalanine, B – biomass, 2-PE – 2-phenylethanol, Eth – ethanol, AA – acetic acid, FA – formic acid, B – 2,3-Butanediol, P – 2-Propanone, PN – 4H-Pyran-4-one, FC – 2-Furancarboxaldehyde, F – 2-Furanmethanol.

Many researchers suggested that the yeast extracts and autolysates can strongly modify wine aroma composition, affecting the volatility of wine aroma compounds [2]. Our data confirmed this statement. In addition to 2-phenylethanol and ethanol *S. cerevisiae* UCM Y-514 and UCM Y-524 produced other aroma compounds such as acetic acid, formic acid, 2,3-butanediol, 2-propanone, 4H-pyran-4-one, 2-furancarboxaldehyde, 2-furanmethanol. Volatile compounds were detected in higher concen-

trations at the presence of cultivating media 9–18% sucrose and 0.75–1.5% yeast extract. This tendency was not observed when we added 0.2 mg/l L-phenylalanine to sucrose and yeast extract. In this case we found present acetic acid, formic acid, and ethyl acetate. This phenomenon could be explained by using the main metabolic pathways via acetyl-CoA and the entry of carbon into the central metabolism via succinyl-CoA [12, 13].

Table 2

ANOVA for biomass yield (Y1), 2-phenylethanol (Y2) and ethanol production (Y3) by *S. cerevisiae* UCM Y-524 as a function of sucrose (X_1), yeast extract (X_2) and L-phenylalanine (X_3) concentrations and their interactions

Factors	Sum of squares	Degrees of freedom	Mean sum of squares	F value	p
for biomass yield					
YE (X_2)	1.237531	1	1.237531	74.85068	0.013098
Lph (X_3^2)	0.386508	1	0.386508	23.37749	0.040214
Total	*2.416543*	*14*			
for 2-phenylethanol production					
Su (X_1)	0.755050	1	0.755050	1078.643	0.000926
Su (X_1^2)	0.508898	1	0.508898	726.997	0.001373
YE (X_2)	0.115824	1	0.115824	165.463	0.005989
YE (X_2^2)	0.198307	1	0.198307	283.295	0.003511
Lph (X_3^2)	0.102052	1	0.102052	145.788	0.006789
$X_1 \cdot X_2$	0.185761	1	0.185761	265.373	0.003747
$X_1 \cdot X_2^2$	0.301088	1	0.301088	430.126	0.002317
$X_1^2 \cdot X_2$	0.097682	1	0.097682	139.546	0.007090
Total	*2.710487*	*14*			
for ethanol production					
Su (X_1^2)	53.915	1	53.915	56.9744	0.017103
YE (X_2)	409.694	1	409.694	432.9425	0.002302
YE (X_2^2)	53.774	1	53.774	56.8254	0.017146
Lph (X_3)	26.404	1	26.404	27.9024	0.034021
Lph (X_3^2)	188.694	1	188.694	199.4019	0.004978
$X_1 \cdot X_2^2$	82.497	1	82.497	87.1785	0.011277
$X_1^2 \cdot X_2$	22.145	1	22.145	23.4012	0.040175
$X_1^2 \cdot X_3$	52.326	1	52.326	55.2958	0.017608
$X_2 \cdot X_3$	113.423	1	113.423	119.8589	0.008240
Total	*1027.689*	*14*			

* Su – sucrose concentrations, YE – yeast extract concentrations, Lph – L-phenylalanine concentrations;
** X_1, X_2, X_3 – linear term, X_1^2, X_2^2, X_3^2 – quadratic term;
*** $p \leq 0.05$.

The profiles for adjusted response for factors indicate that the sucrose and yeast extract concentrations were 13.5% and 1.5% respectively, without L-phenylalanine giving an optimal *S. cerevisiae* UCM Y-514 biomass yield 2.5 g/l. The optimal *S. cerevisiae* UCM Y-524 biomass yield of 2.53 g/l gives high yeast extract and L-phenylalanine concentrations. Profiles for adjusted values for optimum 2-phenylethanol production 1.21 mg/ml were both for *S. cerevisiae* UCM Y-514 and UCM Y-524 with yeast extract concentrations of 0.75% and L-phenylalanine concentration of 0.2 mg/l (Fig. 1).

The adjusted values of optimal ethanol production show that the optimum concentrations were obtained at 22.39 and 21.3 mg/ml respectively for *S. cerevisiae* UCM Y-514 and UCM Y-524. The adjusted profiles indicate that the yeast extract concentrations maintained the same 1.5% for *S. cerevisiae* UCM Y-514 and UCM Y-524, while the sucrose and L-phenylalanine concentrations did not differ significantly.

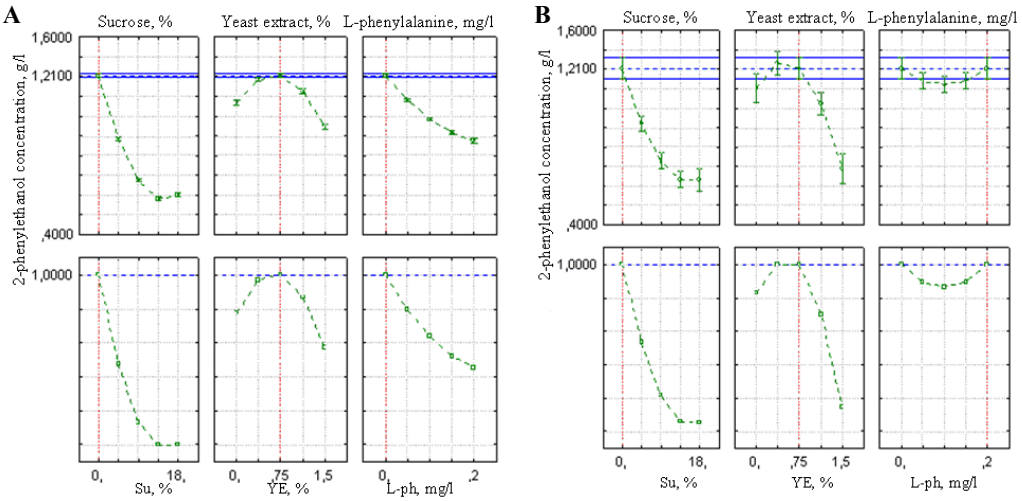

Fig. 1. Profiles for adjusted values of optimal 2-phenylethanol production by *S. cerevisiae* UCM Y-514 (A) and *S. cerevisiae* UCM Y-524 (B) with respect to different factors

In summary, based on the data obtained in the present study, yields of the biomass, 2-phenylethanol and ethanol production were optimized using a statistically designed experiment. The optimized conditions for maximum 2-phenylethanol syntheses were determined to be 5% sucrose, 0.75% yeast extract and 0.2 mg/l L-phenylalanine. The optimized conditions for maximum ethanol syntheses were set at 9—13.5% sucrose, 1.5% yeast extract and 0.15—0.2 mg/l L-phenylalanine. The results showed that *S. cerevisiae* UCM Y-514 and UCM Y-524 were capable of producing up to 1.21 g/l of 2-phenylethanol and up to 22.39 g/l of synthesized ethanol. The high biomass yield completely confirmed that in these concentrations alcohols in cultivation medium are not toxic to yeast.

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СИНТЕЗ 2-ФЕНИЛЭТАНОЛА И ЭТАНОЛА ДРОЖЖАМИ *SACCHAROMYCES CEREVISIAE*

Реферат

Настоящая работа сфокусирована на определении синтеза 2-фенилэтанола и этанола с использованием методов статистического анализа на основании модели Бокса-Бенкена при действии различных концентраций сахарозы, дрожжевого экстракта и L-фенилаланина. Влияние каждого фактора, также как и их комбинаций, оценено методом ANOVA. *S. cerevisiae* УКМ Y-514 и УКМ Y-524 при самом высоком рассчитанном вероятностном уровне синтезируют до 1,21 г/л 2-фенилэтанола. При этом уровень синтеза этанола достигает 22,39 г/л. Выведены уравнения регрессии на основании статистического анализа влияния трёх основных эффектов, получены коэффициенты регрессии и коэффициенты детерминации.

Ключевые слова: *S. cerevisiae*, 2-фенилэтанол, этанол, оптимизация, статистический анализ.

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СИНТЕЗ 2-ФЕНІЛЕТАНОЛУ ТА ЕТАНОЛУ ДРІЖДЖАМИ *SACCHAROMYCES CEREVISIAE*

Реферат

Дане дослідження спрямоване на визначення продукування 2-фенілетанолу і етанолу з використанням методів статистичного аналізу на основі моделі Бокса-Бенкена за дії різних концентрацій сахарози, дріжджового екстракту і L-фенілаланіну. Вплив кожного фактору, також і їх комбінації, оцінені методом ANOVA. *S. cerevisiae* УКМ Y-514 і УКМ Y-524 за найвищого розрахованого ймовірного рівня синтезують до 1,21 г/л 2-фенілетанолу. При цьому рівень синтезу етанолу досягає 22,39 г/л. Виведені рівняння регресії на основі статистичного аналізу впливу трьох основних ефектів, отримані коефіцієнти регресії і коефіцієнти детермінації.

Ключові слова: *S. cerevisiae*, 2-фенілетанол, етанол, оптимізація, статистичний аналіз.