Optimization of bioethanol production from soybean molasses using different strains of *Saccharomyces cerevisiae*

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Abstract

Bioethanol technology represents an important scientific research area because of the high market value and wide availability of its primary and by-products. Worldwide interest in utilizing bioethanol as a renewable and sustainable energy source has significantly increased in the last few years due to limited reserves of fossil fuels and concerns about climate change. Therefore, improvement of the bioethanol production process is a priority research field at the international scale, due to both economic and environmental reasons. The aim of this study was to optimize production of bioethanol from soybean molasses based media using response surface methodology. Three different strains of the yeast *Saccharomices cerevisiae*, commercially available in dried form, were used as production microorganisms, and the best results were obtained by using dried baker's yeast. The results of optimization of alcoholic fermentation using dried baker's yeast indicate that the highest value of the overall desirability function (0.945) is obtained when the initial sugar content is 18.10 % (w/v) at the fermentation time of 48.00 h. At these conditions the model predicts that bioethanol concentration is 8.40 % (v/v), yeast cell number $2.21 \cdot 10^8$ cells/mL and the residual sugar content is 0.35 % (w/v).

Keywords: *bioethanol; Saccharomyces cerevisiae; soybean molasses; response surface methodology; desirability function*

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1. INTRODUCTION

The greatest challenges for society nowadays are to meet the growing demand for energy in a sustainable manner [1]. Considering that conventional energy sources cannot satisfy the growing energy needs due to limited quantities, while their use has a negative impact on the environment causing increased greenhouse gas emissions, it is necessary to develop and use alternative energy sources. Bioethanol is one of the most promising and environmentally friendly renewable energy sources that has become an attractive alternative biofuel made from renewable raw materials rich in carbohydrates. Pure hydrous ethanol or anhydrous ethanol mixed with gasoline is widely used as a transport fuel [2,3].

Fermentation of the appropriate raw material into bioethanol is performed by microorganisms, traditionally by yeasts, although some types of bacteria could also be used. After the bioprocess, bioethanol is separated from the broth by distillation, rectification, pervaporation, membrane filtration or molecular sieves [4]. The choice of the raw material to be used for bioethanol production depends on the geographical area and conditions of agricultural production, which can vary from season to season [5]. A wide range of raw materials can be used, such as sugar-based (sugar beet or sugarcane juice and molasses), starch-based (corn and wheat) and cellulosic (bagasse and wood) resources. Specifically, sugar-based raw materials contain fermentable sugars that can be readily utilized, thus representing an ideal substrate for bioethanol production, due to the fact that starch and cellulosic substances need to be pre-treated before they can be converted into fermentable sugars [6]. Currently, all commercially produced bioethanol is made from sugarcane and corn, but these two raw materials are not sufficient to replace huge amounts of conventional fuels currently consumed as well as to meet the rising demand for bioethanol, which is why it is important to search for new bioethanol raw materials [2]. The alcoholic fermentation process is well known, but production costs remain the key obstacle for a

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wider use of bioethanol as a fuel, which indicates the importance to use economical carbon sources for commercial bioethanol production [7].

Soybean is one of the world's principal agricultural products due to its high productivity, adaptability, and nutritional qualities [8]. The Republic of Serbia is one of the largest producers of oil crops in Europe – the average soybean yields in Serbia were on par with the average global yield with 385,514 tons produced in 2013 [9]. During the soybean processing, more precisely during the production of the soybean protein concentrate from de-oiled soybean meal, soybean molasses is generated as a by-product with the fraction of 23 %. So it can be concluded that this crop and its residue represent an available raw material in the Republic of Serbia [7]. Soybean molasses is a sweet-sour brown viscous liquid obtained during the extraction of soybean proteins, which contains a high content of sugar (57 % dry weight), nitrogen, and other macro- and micronutrients. The sugars in molasses that can be converted into bioethanol are sucrose, glucose, and fructose, while approximately 47 % of the sugars in soybean molasses cannot be fermented by *Saccharomyces cerevisiae* [8, 10].

Several factors affect the fermentation process as well as the bioethanol yield such as the production microorganism strain, fermentation time and initial sugar concentration [11]. Therefore, it is important to use optimal values of these parameters, for each selected production microorganism, in order to obtain highest possible amounts of the desired product. Optimization by using the experimental design methodology and statistical approaches is a common practice in biotechnology [12]. Response surface methodology (RSM) is a statistical procedure frequently used for optimization studies that uses quantitative data from appropriately designed experiments to determine and simultaneously solve multivariate problems. The equations describe effects of the test variables on predicted responses, providing the assessment of relationships between test variables and predicting the combined effect of all test variables on the response. This approach enables efficient exploration of a process or a system [13] and is extensively used for optimization of bioethanol production [6, 14-18].

The aim of this study was the application of RSM for optimization of the fermentation time and initial sugar concentration in the bioethanol production from soybean molasses by different strains of *Saccharomyces cerevisiae*. Additionally, fermentation efficiencies under optimal conditions were compared and the most suitable yeast strain was selected.

2. EXPERIMENTAL

2. 1. Soybean molasses

Soybean molasses obtained from a domestic soybean-processing factory in the concentrated form (~ 60 % dry matter) was used as a raw material for fermentation media preparation. The hydrolyzed molasses (as proposed by Romão et al. [8]) was diluted with water to result in total sugar concentrations of 10, 15 and 20 % (w/v). The prepared media were adjusted to pH 5.0 before sterilization by autoclaving at 121°C and 2.1 bar for 20 min.

2. 2. Production microorganisms

Three different strains of *Saccharomyces cerevisiae* commercially available in dried form were used in this research: dried distiller's yeast (Lallemand Inc., Rexdale, Ontario, Canada), dried wine yeast (Lallemand Inc., Rexdale, Ontario, Canada) and dried baker's yeast (Alltech, Senta, Serbia). In order to rehydrate dried yeasts and metabolically acclimatize the cells prior to the fermentation, the yeasts were suspended in a small quantity of media under aerobic conditions for 2 h (temperature of 30°C, agitation rate of 200 rpm) and then introduced to the rest of the fermentation medium.

2. 3. Bioethanol production

The experiments were performed in 300 mL Erlenmeyer flasks containing 100 mL of the fermentation medium. The sterile medium was inoculated with the yeast suspension to provide the initial cell concentration of 10^8 cells/mL. Bioethanol production was carried out in batch mode under anaerobic conditions at the temperature of 30°C and the agitation rate of 150 rpm on a laboratory shaker (KS 4000i control, Ika® Werke, Germany) for 24 h, 36 h and 48 h.

2. 4. Analytical methods

Bioethanol concentration in distillates of the fermented media was determined by gas chromatography using a HP 5890 Series II GC (Agilent Technologies Inc, Santa Clara, CA, USA) equipped with a flame ionization detector, a Carbowax 20 M column at 85°C and the carrier gas was helium. Injector and detector temperatures were maintained at 150°C.

Cell number in the fermented medium was determined by using a Neubauer Haemocytometer under 400 x magnifications using an optical microscope (Wild M20, Heerbrugg, Gais, Switzerland).

Residual sugar content in the cell-free fermented medium, obtained by centrifugation at 4000 rpm for 15min (HettichRotina 380 R, Germany), was determined by using high pressure liquid chromatography (HPLC). The samples

were filtered through a 0.45 µm nylon membrane (Agilent Technologies Inc, Germany) and then analyzed. The HPLC instrument (Thermo Scientific DionexUltiMate 3000 series, country) was equipped with a pump HPG-3200SD/RS, autosampler WPS-3000(T)SL (10 µL injection loop), column ZORBAX NH2 (250 × 4.6 mm, 5 µm) (Agilent Technologies Inc, USA) and a detector RefractoMax520 (ERC Inc, Japan). The acetonitrile solution (75 % v/v) was used as the eluent at the flow rate of 1.2 mL/min and the elution time of 20 min at the column temperature of 25 °C.

2. 5. Statistical analysis

All experiments in this study were carried out in triplicate and the results were averaged. The obtained data was statistically processed by the analysis of variance at the significance level of $\alpha = 0.05$ and used for mathematical modelling. A second-degree polynomial model was applied in order to define relations describing the influence of varied factors (X_1 and X_2) on selected responses ($Y_{1\text{-}9}$), generally having the following form:

$$Y_{1-9} = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 \quad 1)$$

where b_0 represents the intercept, b_1 and b_2 represent linear coefficients, b_{11} and b_{22} represent quadratic coefficients, and b_{12} represents the interaction effect of the factors. The factors were the initial sugar content in the medium (X_1: 10-20 % w/v, interval value 5 % w/v) and the fermentation time (X_2: 24-48h, interval value 12 h), while the responses were the bioethanol concentration (Y_1, Y_4 and Y_7, % v/v), the yeast cell number (Y_2, Y_5 and Y_8, cells/mL) and the residual sugar content (Y_3, Y_6 and Y_9, % w/v). Adequacy of the defined mathematical models was evaluated by the coefficient of determination (R^2), while the significance of each regression coefficient in the obtained models was determined by the *p*-value where $p < 0.05$ was considered as statistically significant. Statistically significant values are marked with asterisks in Tables 1, 3 and 5.

Statistical analyses of the experimental results, definition of the mathematical models and generation of response surface plots were performed by using Statistica software v. 13.2 (Dell Inc., USA). Defined mathematical equations and the method of desirability function were used to determine optimal values of the examined factors in the software package Design-Expert 8.1. (Stat-Ease, Inc., USA)

3. RESULTS AND DISCUSSION

In order to optimize the initial sugar concentration and fermentation time for bioethanol production by different strains of *Saccharomices cerevisiae* on soybean molasses - based media, experiments were performed in triplicate and under identical conditions.

3. 1. Bioethanol production by distiller's yeast

Results of bioethanol fermentation by using dried distiller's yeast were fitted with a second-order polynomial model, and the results of the statistical analysis and mathematical modelling for selected responses are shown in Table 1 and Table 2.

Table 1.Bioethanol production by distiller's yeast: regression equation coefficients and their significance for selected responses

Effect	Y_1		Y_2		Y_3	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
			Intercept			
b_0	-3.9245	0.2230	-0.0122	0.9791	5.1247	0.3152
			Linear			
b_1	-0.1863	0.4947	0.1863	0.0188*	0.9971	0.0881
b_2	0.3048	0.0557	0.0177	0.3659	-0.3965	0.0975
			Quadratic			
b_{11}	0.0015	0.8515	-0.0051	0.0269*	0.0059	0.6693
b_{22}	-0.0053	0.0277*	-0.0002	0.4387	0.0068	0.0522
			Interaction			
b_{12}	0.0107	0.0169*	0.0004	0.3985	-0.0172	0.0185*

*Significant at 95 % confidence level ($p<0.05$); Y_1 - bioethanol concentration, Y_2 - yeast cell number, Y_3 - residual sugar content.

Table 1 shows that the interaction coefficient of the initial sugar content and fermentation time as well as the square coefficient of fermentation time in the bioethanol concentration model are significant, which confirms that both parameters are important for this bioprocess [19,20]. The positive interaction between these two independent variables indicates that there is a synergetic effect of the initial sugar concentration and fermentation time on bioethanol

production. In the model for yeast cell number, the linear regression coefficient and the square coefficient of the initial sugar content are significant, which can be explained by the fact that the initial carbon source concentration has a direct effect on microbial cell growth as well as on its viability and metabolic activity [21,22]. In the model for the residual sugar content, the interaction coefficient of the initial sugar content and fermentation time is significant. Considering that this coefficient has a negative sign, the unused amount of sugar can be regulated by reducing the initial sugar concentration or by increasing the fermentation time. The first option is not recommended because fermentation of media with a decreased sugar content results in the reduction of product yield as well as the increase of production costs since it is not profitable to distil media with low bioethanol concentration [23].

ANOVA results for the selected responses are presented in Table 2. The high value of the coefficient of determination indicates a very good agreement of the applied polynomial model and the experimental results, but the obtained models do not cover 1.1, 1.3 and 0.8 % of variations in results for bioethanol concentration (Y_1), yeast cell number (Y_2) and residual sugar content (Y_3) obtained in media fermented by dried distiller's yeast, respectively. Additionally, the significance of developed models at the 95 % confidence level was evaluated based on the F-values, and the obtained values of 247.347, 3452.738 and 94.343 for bioethanol concentration, yeast cell number and residual sugar content, respectively, imply that models for selected responses are significant.

Table 2.Bioethanol production by distiller's yeast: analysis of variance (ANOVA) of the modeled responses

Response	Source								
	Residual			Model					
	DF	SS	MS	DF	SS	MS	F-value	p-value	R^2
Y_1	3	0.212	0.071	6	105.936	17.489	247.347	0.000395	0.989
Y_2	3	0.006	0.002	6	40.742	6.790	3452.738	0.000008	0.987
Y_3	3	0.587	0.196	6	566.056	94.343	482.556	0.000146	0.992

DF – degree of freedom, SS – sum of squares, MS – mean squares.
Y_1– bioethanol concentration, Y_2– yeast cell number, Y_3– residual sugar content.

In order to better understand interactions of variables that influence bioethanol concentrations obtained by fermentation of media based on soybean molasses using dried distiller's yeast, a response surface plot is generated and shown in Figure 1.

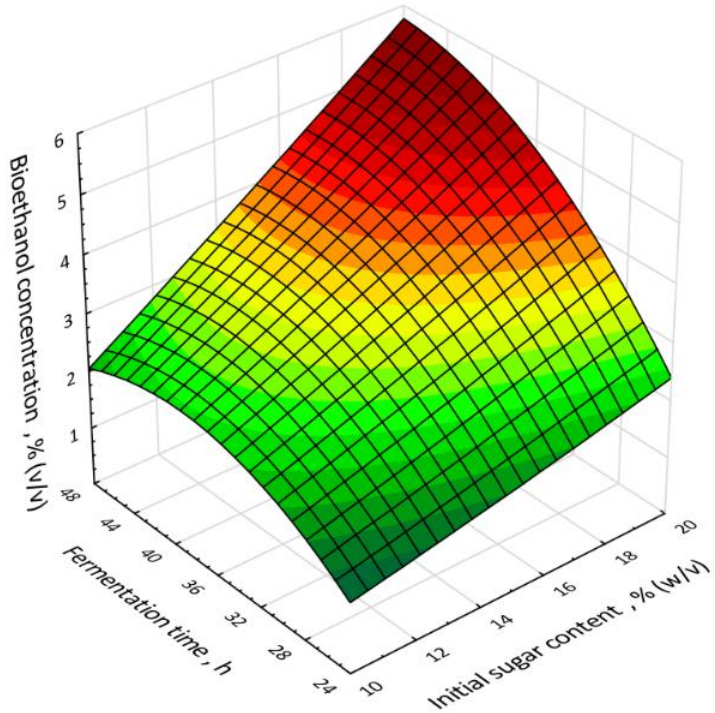

Figure 1. Effects of the initial sugar content and fermentation time on bioethanol concentration in media fermented by distiller's yeast

Figure 1 shows that at shorter fermentation durations (24-32 h), increase in the initial sugar concentration in the media almost does not affect the increase rate in bioethanol concentration. However, when fermentation time is longer

(36-48 h), higher concentrations of the initial sugar (15-20 %, w/v) induce the increase in the obtained bioethanol concentration. The maximum bioethanol concentration of about 6 % (v/v), at the applied experimental conditions, is obtained at the maximum value of the initial sugar content (20 %, w/v) and the maximal fermentation time (48 h).

3. 2. Bioethanol production by wine yeast

Results of statistical analysis and mathematical modelling of experimental data obtained for media fermented by dried wine yeast are shown in Tables 3 and 4. Table 3 shows that the models for bioethanol concentration and yeast cell number contain significant linear and square coefficients of the initial sugar content, while the model for residual sugar content contains significant square coefficients of the initial sugar content and fermentation time. The obtained results are similar to the results of the previous experimental series. The only difference is in the factors that significantly affect the bioethanol production. In this case, the influence of the initial sugar content on bioethanol concentration in the fermented medium is more pronounced as compared to the fermentation time, which is probably a result of using another yeast strain [11].

Table 3. Bioethanol production by wine yeast: regression equation coefficients and their significance for selected responses

Effect	Y_4		Y_5		Y_6	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
			Intercept			
b_0	-6.8233	0.1478	0.1072	0.8242	9.3592	0.1515
			Linear			
b_1	1.1543	0.0396*	0.1629	0.0294*	-1.1481	0.0874
b_2	-0.0936	0.5449	0.0250	0.2434	0.3390	0.1742
			Quadratic			
b_{11}	-0.0330	0.0496*	-0.0052	0.0276*	0.0585	0.0266*
b_{22}	0.0033	0.1664	-0.0002	0.0476	-0.0084	0.0432*
			Interaction			
b_{12}	-0.0018	0.5895	0.0002	0.6006	0.0028	0.5517

*Significant at 95 % confidence level (p<0.05); Y_4 - bioethanol concentration, Y_5 - yeast cell number, Y_6 - residual sugar content.

Table 4.Bioethanol production by wine yeast: analysis of variance (ANOVA) of the modeled responses

Response	Source								
	Residual			Model					
	DF	SS	MS	DF	SS	MS	F-value	p-value	R^2
Y_4	3	0.400	0.133	6	80.119	13.353	100.081	0.001524	0.973
Y_5	3	0.006	0.002	6	38.271	6.379	3024.054	0.000009	0.977
Y_6	3	0.773	0.257	6	745.711	124.285	482.429	0.000146	0.994

DF – degree of freedom, SS – sum of squares, MS – mean squares.
Y_4 - bioethanol concentration, Y_5 - yeast cell number, Y_6 - residual sugar content.

In Table 4, the values of coefficients of determination indicate a very good agreement of the applied polynomial model with experimental results. However, 2.7 %, 2.3 % and 0.6 % of variations in the results for bioethanol concentration (Y_4), yeast cell number (Y_5) and residual sugar content (Y_6) could not be explained by the developed models, which describe the fermentation process performed by dried wine yeast. In addition, the model F-values of 100.081, 3024.054 and 482.429 for the bioethanol concentration, yeast cell number and residual sugar content, respectively, indicate that the mathematical models for analysed responses are significant.

Effects of the initial sugar concentration and fermentation time on bioethanol concentration in media fermented by dried wine yeast are shown in Figure 2. Based on the results shown on this response surface plot it can be noted that at the lowest applied fermentation times (24-36 h) and in the entire range of initial sugar concentrations, the obtained bioethanol concentrations are the lowest (up to 3 %, v/v). However, with further increase in the fermentation time in the entire range of initial sugar contents, there is a noticeable increase in the obtained bioethanol concentration. The maximal values of the obtained bioethanol concentrations are around 5 % (v/v) when the sugar concentration was initially between 14 % (w/v) and 18 % (w/v) and the fermentation time was about 48 h.

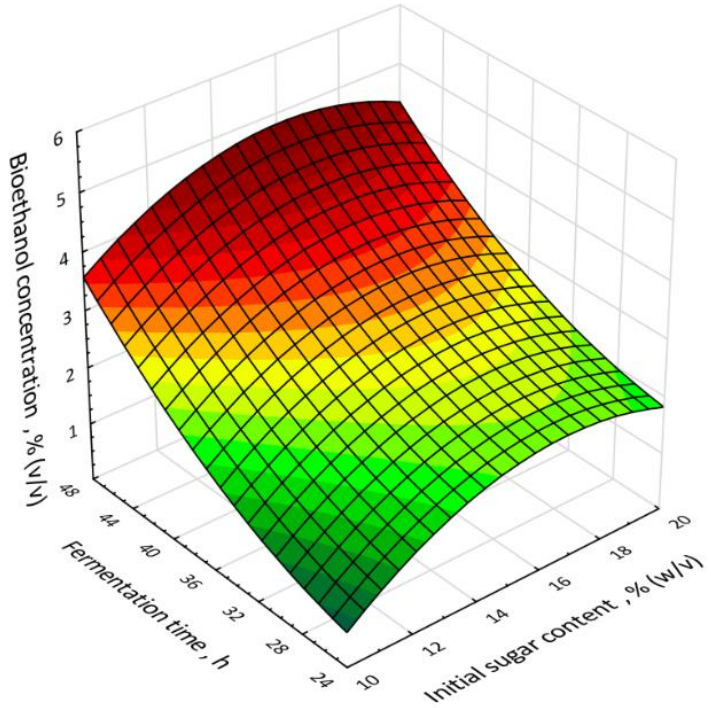

Figure 2. Effects of the initial sugar content and fermentation time on bioethanol concentration in media fermented by wine yeast

3. 3. Bioethanol production by baker's yeast

Results of statistical analysis and mathematical modelling of experimental data obtained for media fermented by dried baker's yeast are shown in Tables 5 and 6. For the selected responses, statistical analysis results (Table 5) show that the model for bioethanol concentration contains a significant square regression coefficient of the initial sugar content as well as the coefficient of the interaction of the initial sugar content and fermentation time, while models for the yeast cell number and the residual sugar content contain only a significant interaction coefficient of the initial sugar content and fermentation time. Positive values of the interaction coefficients in models for bioethanol concentration and cell number suggest that the yeast strain used in this research series requires media with higher sugar contents at longer fermentation times to grow and to produce the desired product in sufficient amounts.

Results of the ANOVA test are reported in Table 6. Based on the relatively high values of the coefficient of determination obtained for all analysed responses it can be concluded that there is a good agreement of the second-order polynomial model with experimental data. Therefore, only 0.6 %, 1.9 % and 1.9 % of variations in the results for bioethanol concentration (Y_7), yeast cell number (Y_8) and residual sugar content (Y_9), respectively, could not be explained by the defined models for alcoholic fermentation using dried baker's yeast. The model F-values (1241.409 for the bioethanol concentration, 475.088 for the yeast cell number and 50.163 for the residual sugar content) show that the models for selected responses are significant.

Table 5. Bioethanol production by baker's yeast: regression equation coefficients and their significance for selected responses

Effect	Y_7		Y_8		Y_9	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
			Intercept			
b_0	-1.7983	0.4461	1.3183	0.2329	0.9236	0.8110
			Linear			
b_1	0.4507	0.0969	0.0051	0.9552	0.1575	0.6676
b_2	0.0713	0.4406	-0.0345	0.3915	-0.0715	0.6411
			Quadratic			
b_{11}	-0.0124	0.0304*	-0.0024	0.4206	0.0239	0.1047
b_{22}	-0.0029	0.0727	-0.0001	0.9698	0.0035	0.1481
			Interaction			
b_{12}	0.0146	0.0038*	0.0038	0.0158*	-0.0183	0.0094*

*Significant at 95 % confidence level (p<0.05); Y_7 - bioethanol concentration, Y_8 - yeast cell number, Y_9 - residual sugar content.

Table 6. Bioethanol production by baker's yeast: analysis of variance (ANOVA) of the modeled responses

Response	Source								
	Residual			Model					
	DF	SS	MS	DF	SS	MS	F-value	*p*-value	R^2
Y_7	3	0.137	0.046	6	339.136	56.539	1241.409	0.000035	0.994
Y_8	3	0.025	0.008	6	24.028	4.005	475.088	0.000149	0.981
Y_9	3	0.405	0.135	6	40.627	6.771	50.163	0.004239	0.981

DF – degree of freedom, SS – sum of squares, MS – mean squares.
Y_7 - bioethanol concentration, Y_8 - yeast cell number, Y_9 - residual sugar content.

Effects of the initial sugar concentration and fermentation time on bioethanol concentration in media fermented by baker's yeast can be analysed by response surface presented in Figure 3. For shorter fermentation times (24-32 h), the increase in sugar content does not affect the increase rate in the bioethanol concentration. However, for longer fermentation times (36-48 h) and with the increase in the initial sugar content, the concentration of the desired product is greatly increased. The response surface shows that the maximum bioethanol concentration (~9 %, v/v) is obtained after 48 h of fermentation with the highest initial sugar concentrations applied (~20 %, w/v).

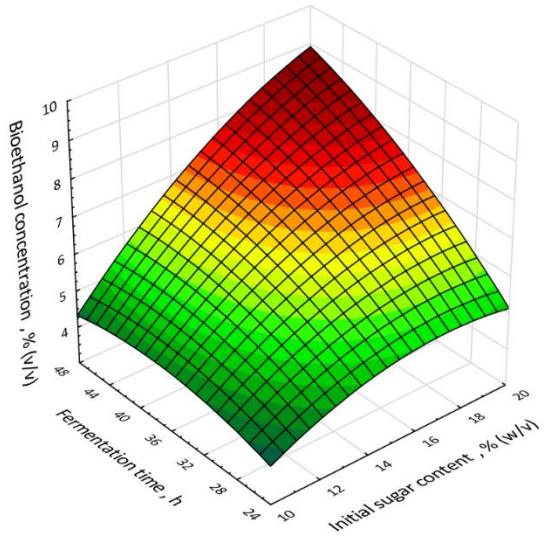

Figure 3. Effects of the initial sugar content and fermentation time on bioethanol concentration in media fermented by baker's yeast

3.4. Optimization of bioethanol production

The final goal of the response surface methodology is optimization of the investigated bioprocess using the desirability function method in the case of a process with two or more responses. Thus, the developed models can be used for simulations and optimization. The general approach consists of converting individual responses into individual desirability functions, which values range between 0 and 1. The value "0" of the individual desirability function represents the lowest value, while the "1" represents the highest value of the observed response. The overall desirability function equals to the geometric average of all individual desirability functions [24].

The desirability function method was used in the present study to determine the optimal initial sugar content and fermentation time for the production of bioethanol on media based on soybean molasses using three different strains of yeast *Saccharomyces cerevisiae*. The maximum bioethanol production with the least amount of residual sugars in the fermented media was defined as the optimization aim with respect to the economic and environmental significance of these parameters in the biotechnological production. The success and cost-effectiveness of a bioprocess is assessed based on the amount of the obtained desired product, while residual nutrients represent economic losses. Additionally, residual nutrients add a significant organic load to the effluents generated by the bioprocess, which requires additional treatment to prevent a negative environmental impact. Therefore, the residual nutrient content must be controlled and kept at very low levels, amounting to up to 0.5 % (w/v) of the total sugar [23].

Values of the overall desirability function were obtained as a result of optimization, limited by minimizing and maximizing the selected responses, as shown in Table 7. Figure 4 shows the bioethanol concentration as a function of the values of varied parameters for all three used yeast strains. Based on the obtained contour diagrams, it can be seen that the change in bioethanol content is in accordance with previously defined mathematical models.

Based on the results presented in Table 7 it can be noticed that for bioethanol production by using dried distiller's yeast, the desirability function has the highest value (0.755) at the initial sugar content of 18.49 % (w/v) and fermentation time of 46.50 h. At these conditions, the model predicts the bioethanol concentration of 5.19 % (v/v), accumulation of $2.41 \cdot 10^8$ yeast cells/mL and detection of 7.01% (w/v) residual sugar.

When alcoholic fermentation is performed with dried wine yeast, the maximum value of the overall desirability function of 0.898 is obtained at the initial sugar content of 13.99 % (w/v) and fermentation time of 48.00 h (Table 7). In this case, the residual sugar in the fermentation broth is 3.53 % (w/v), while the achieved bioethanol concentration is 4.66% (v/v) at the final yeast cell number of $2.29 \cdot 10^8$ cells/mL.

Finally, the optimization results for bioethanol production using dried baker's yeast (Table 7) indicate that the value of the overall desirability function is the highest (0.945) at the initial sugar content of 18.10 % (w/v) and the fermentation time of 48.00 h. At these conditions the model predicts the following response values: the bioethanol concentration of 8.40 % (v/v), the yeast cell number of $2.21 \cdot 10^8$ cells/mL and the residual sugar content of 0.35 % (w/v).

The obtained values show that the best fermentation results (*i.e.* the highest bioethanol concentration and the lowest residual sugar content) are obtained by using dried baker's yeast as the production microorganism in the bioethanol production from soybean molasses based media. However, previous experiments with media based on by-products of sugar beet processing, including molasses, suggested that there were insignificant differences in bioethanol production when using these three yeast strains [25].

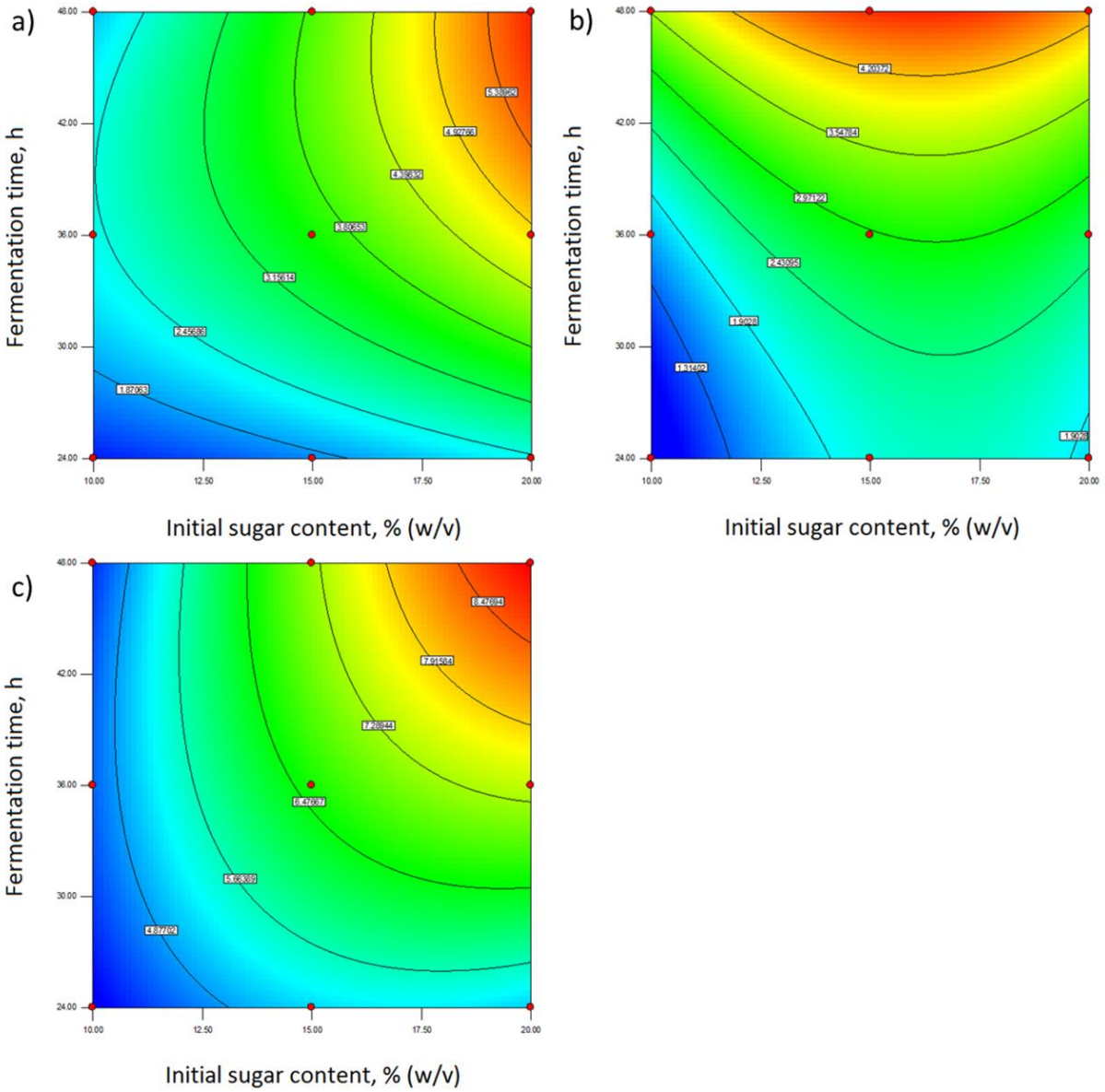

Figure 4. Contour plots for the bioethanol concentration in media fermented by distiller's yeast (a), wine yeast (b) and baker's yeast (c)

Table 7.Optimization results for bioethanol production using different strains of Saccharomyces cerevisiae

Factors and responses	Goals	Predicted value
Dried distiller's yeast		
Initial sugar content, % (w/v)	in range	18.49
Fermentation time, h	in range	46.50
Bioethanol concentration, % (v/v)	maximize	5.19
Yeast cell number, 10^8 cells/mL	in range	2.41
Residual sugar content, % (w/v)	minimize	7.01
Overall desirability function	0,755	
Dried wine yeast		
Initial sugar content, % (w/v)	in range	13.99
Fermentation time, h	in range	48.00
Bioethanol concentration, % (v/v)	maximize	4.66
Yeast cell number, 10^8 cells/mL	in range	2.29
Residual sugar content, % (w/v)	minimize	3.53
Overall desirability function	0.898	
Dried baker's yeast		
Initial sugar content, % (w/v)	in range	18.10
Fermentation time, h	in range	48.00
Bioethanol concentration, % (v/v)	maximize	8.40
Yeast cell number, 10^8 cells/mL	in range	2.21
Residual sugar content, % (w/v)	minimize	0.35
Overall desirability function	0.945	

3. 5. Validation of optimization results and selection of the yeast strain

In order to validate the developed models, three confirmation experiments were carried out simultaneously using the optimal values of the initial sugar content and fermentation time for each of the used strains of *Saccharomyces cerevisiae*, and the obtained results are shown in Table 8.

Table 8. Results of bioethanol production under optimal conditions using three investigated strains of Saccharomyces cerevisiae

Run	Initial sugar content, % (w/v)	Fermentation time, h	Bioethanol concentration, % (v/v)	Yeast cell number, 10^8 cells/mL	Residual sugar content, % (w/v)
			Dried distiller's yeast		
1	18.50	46.50	5.08	2.38	7.22
2	18.50	46.50	4.75	2.35	7.44
3	18.50	46.50	5.53	2.43	6.86
			Dried wine yeast		
1	14.00	48.00	5.02	2.35	3.12
2	14.00	48.00	4.17	2.08	4.15
3	14.00	48.00	4.55	2.21	3.84
			Dried baker's yeast		
1	18.00	48.00	8.71	2.36	0.28
2	18.00	48.00	7.83	2.07	0.47
3	18.00	48.00	8.39	2.16	0.39

Based on the obtained results, it can be seen that experimental values are in excellent accordance with the predicted values for all modelled responses. The highest concentration of bioethanol was obtained by using dried baker's yeast (8.31±0.45 %, v/v), while somewhat lower concentrations of the product were obtained by the other two yeast strains (5.12±0.39 %, v/v for dried distiller's yeast and 4.58±0.43 %, v/v for dried wine yeast). The literature data indicate that the use of commercial types of *Saccharomyces cerevisiae* in fermentation of media based on sugar beet and sugar cane molasses, resulted in bioethanol concentrations between 7 and 9 % (v/v) [26]. Therefore, it is evident that commercial dried baker's yeast used in the present research is equally viable. It is also important to note that baker's yeast in fresh or dried form is traditionally used as a starter culture in bioethanol production due to its low cost, wide availability, resistance to bacterial contamination and simple usage [27, 28]. In industrial bioethanol production the yeast biomass

is recycled in order to reduce the costs and improve the process productivity. Still the differences among yeast strains observed in the present work may be valid also at the industrial level, which should be further investigated.

Additionally, results for bioethanol concentration in fermented media and data for sugar consumption were used for calculation of the product yield. The bioethanol yield at the applied experimental conditions ranged in the interval of 0.350-0.372 g/g, which is about 70 % of the theoretical yield [23]. These results are similar to the results reported in literature for alcoholic fermentation of media containing soybean molasses [7, 10]. The yeast strains applied in the present study were also used for bioethanol production from media based on effluents generated during sugar beet processing, including molasses, where somewhat higher values for the bioethanol concentration and bioethanol yield were achieved [29]. These differences are expected due to high contents of non-fermentable sugars, nitrogen, and other macro- and micronutrients in soybean molasses that was used in the present study [8].

Still, the obtained results indicate that soybean molasses represents an excellent raw material for bioethanol production. Considering that soybean molasses contains non-fermentable sugars by the used microorganism together with fermentable sugars, it is necessary to hydrolyse this raw material prior to fermentation. Although acid hydrolysis is commonly performed, enzyme hydrolysis is more efficient. Intra and extracellular invertase are enzymes produced by *Saccharomyces cerevisiae*, the latter of which enables cleaving of sucrose into glucose and fructose that can be converted into bioethanol. However, an additional enzyme, specifically α-galactosidase, is necessary for the metabolism of complex sugars contained in soybean molasses (stachyose and raffinose). Research has shown that bioethanol production can be increased if the raw material is hydrolysed by this enzyme [30].

4. CONCLUSION

The results obtained in this study indicate a significant potential of using soybean molasses for biotechnological production of bioethanol. Additionally, it is shown that all used yeast strains are viable, but that the highest concentration of the desired product is obtained by using dried baker's yeast. Defined mathematical models adequately described the examined bioprocess and contributed to better understanding of the effects of the initial sugar content and fermentation time on bioethanol production by using dried distiller's, wine and baker's yeasts at the applied experimental conditions. Further research should focus on examination of the effects of acid and enzyme hydrolysis of the raw material on the overall success of this bioprocess, then optimization of the process parameters in order to increase the bioethanol concentration, as well as on assessment of the bioprocess kinetics as an important step towards scaling up of this biotechnological process.

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SAŽETAK

Optimizacija proizvodnje bioetanola na sojinoj melasi primenom različitih sojeva kvasca *Saccharomyces cerevisiae*

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(Stručni rad)

Tehnologija bioetanola predstavlja značajnu naučnu oblast zbog visoke tržišne vrednosti i široke dostupnosti njenih glavnih i sporednih proizvoda. Interesovanje koje u svetu vlada za bioetanolom kao obnovljivim i održivim izvorom energije značajno je poraslo u poslednjih nekoliko godina što je posledica ograničenih rezervi fosilnih goriva i zabrinutosti zbog klimatskih promena. Stoga je poboljšanje postupka proizvodnje bioetanola prioritetna oblast istraživanja na međunarodnom niovu, kako iz ekonomskih tako i iz ekoloških razloga. Cilj ovih istraživanja je bio optimizacija proizvodnje bioetanola na medijumu čija je osnova sojina melasa primenom postupka odzivne površine. Tri različita soja kvasca *Saccharomices cerevisiae*, komercijalno dostupna u suvoj formi, su korišćena kao proizvodni mikroorganizmi, a najbolji rezultati dobijeni su primenom suvog pekarskog kvasca. Rezultati optimizacije alkoholne fermentacije primenom suvog pekarskog kvaca ukazuju da se najveća vrednost ukupne željene funkcije (0,945) dobija kada je početni sadržaj šećera u medijumu 18,10% (w/v), a vreme fermentacije 48,00 h. Pri ovim uslovima model predviđa da je koncentracija bioetanola 8,40 % (w/v), broj ćelija kvasca $2{,}21 \cdot 10^8$ ćelija ml^{-1}, a rezidualni sadržaj šećera 0,35% (w/v).

Ključne reči: bioetanol, *Saccharomyces cerevisiae*, sojina melasa, postupak odzivne površine, koncept željene funkcije