ISSN 1330-9862
(FTB-2433)

original scientific paper

Bioethanol Production from Raw Juice as Intermediate of Sugar Beet Processing: A Response Surface Methodology Approach

Stevan Popov, Jovana Ranković, Jelena Dodić, Siniša Dodić and Aleksandar Jokić*

University of Novi Sad, Faculty of Technology, Department of Biotechnology and Pharmaceutical Engineering, Bulevar cara Lazara 1, RS-21000 Novi Sad, Serbia

Received: February 1, 2010
Accepted: May 4, 2010

Summary

Response surface methodology (RSM) was used for selecting optimal fermentation time and initial sugar mass fraction in cultivation media based on raw juice from sugar beet in order to produce ethanol. Optimal fermentation time and initial sugar mass fraction for ethanol production in batch fermentation by free *Saccharomyces cerevisiae* cells under anaerobic conditions at the temperature of 30 °C and agitation rate of 200 rpm were estimated to be 38 h and 12.30 % by mass, respectively. For selecting optimal conditions for industrial application, further techno-economic analysis should be performed by using the obtained mathematical representation of the process (second degree polynomial model). The overall fermentation productivity of five different types of yeast was examined and there is no significant statistical difference between them.

Key words: optimization, bioethanol, raw juice, *Saccharomyces cerevisiae*, response surface methodology

Introduction

In recent years, a new round of enthusiasm in biomass and bioenergy has been initiated with the recognition that the global crude oil reserves are limited, and their depletion is occurring much faster than previously predicted. In addition, the environmental deterioration resulting from the overconsumption of petroleum-derived products, especially transportation fuels, is threatening the sustainability of human society. Ethanol, both renewable and environmentally friendly, is believed to be one of the best alternatives, leading to a dramatic increase in its production capacity. Currently, the global ethanol supply is obtained mainly from sugar and starch feedstock (*1*). Less expensive production of sugar from sugarcane indicates that application of sugar beet for bioethanol production has great potential. Also, increased yield and sugar production efficiency have led to a reduction in sugar beet requirements. For these reasons, the majority of existing sugar plants in Europe started simultaneous production of ethanol in refineries built as their extensions. Based on an extensive overview of approaching bioethanol production development in Vojvodina, Serbia, it can be concluded that this region has a large potential for renewable energy, especially energy from biomass – biodiesel and bioethanol (*2-4*). Molasses is commonly used feedstock for bioethanol production. In sugar beet processing, it is a by-product obtained at the end of the process and the cost of its production is considerably higher than the cost of raw juice produced at the beginning by water extraction of sliced sugar beet. Raw juice contains about 15–20 % of dry solids. Its purity ranges between 85 and 90 %, which means that there are about 85–90 % of sugars and 10–15 % of non-sugars in dry matter (*5*). The obtained raw juice can be used either directly for ethanol and sugar production during the sugar beet harvest season, or it can be concentrated in an evaporator and stored for several months (*6*).

*Corresponding author; Fax: ++381 21 450 413; E-mail: johana@uns.ac.rs

In statistics-based approaches, response surface methodology (RSM) has been extensively used in fermentation medium optimization. RSM is a group of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions. In RSM, the experimental responses to the design of experiments are fitted to quadratic function. The number of successful applications of RSM suggests that the second-order relation can reasonably approximate several of the fermentation systems.

The aims of this study are to describe the number of yeast cells, fermentable sugar consumption and ethanol production employing mathematical relationships as well as to find optimal fermentation time and initial sugar concentration for bioethanol production from raw juice in batch fermentation by free *Saccharomyces cerevisiae* cells. Also, the overall fermentation efficiency of different types of yeast (extensively used as starter cultures in various branches of the fermentation industry) are compared.

Materials and Methods

Microorganisms and inoculum preparation

Five different commercial types of *Saccharomyces cerevisiae* were used throughout this research: dried distiller's yeast – DD (Lallemand Inc, Rexdale, Ontario, Canada), dried wine yeast – DW1 (Lallemand Inc, Rexdale, Ontario, Canada), dried wine yeast tolerant of high ethanol concentration – DW2 (Lallemand Inc, Rexdale, Ontario, Canada), dried baker's yeast – DB (Alltech, Senta, Serbia) and fresh baker's yeast – FB (Alltech, Senta, Serbia). In order to rehydrate dried yeasts and metabolically acclimatize the cells prior to fermentation, yeasts were suspended in a small quantity of culture medium under aerobic conditions for 2 h (temperature of 30 °C, agitation rate of 200 rpm) and then introduced to the rest of the culture medium. On the other hand, fresh baker's yeast was also suspended in a small quantity of culture medium and immediately used for inoculation.

Substrates

Raw juices were obtained from sugar factories in Crvenka (SF1) and Senta (SF2), Serbia, during the harvest season 2007/2008. The sugar beet was harvested in Vojvodina. Substrates were kept at –18 °C until use. Raw juices were diluted with distilled water to give a total sugar mass fractions of 5 and 10 % and also used without dilution with a total sugar mass fraction of approx. 13 %, obtained from sugar beet processing technology. The substrates were adjusted to pH=5.0 with 10 % sulphuric acid (by volume).

Fermentation conditions

Fermentations were carried out in a 2-litre bench-scale bioreactor with fermentation medium of 1.5 L. The bench-scale bioreactor with the substrate was sterilized by autoclaving at 121 °C and pressure of 2.2 bar for 30 min. The sterile medium was inoculated to give the initial yeast cell concentration of 10^8 (cells/mL) (approx. 3 g of yeast dry solids per 1000 mL of medium). The fermentation was carried out in batch mode under anaerobic conditions for 48 h, including the time needed for making the suspension, at the temperature of 30 °C and agitation rate of 200 rpm. The samples were taken at predetermined time intervals for analysis.

Measurement techniques

During the fermentation, samples for analysis were taken at the beginning and at the predetermined time intervals: 4, 8, 12, 24, 30, 36 and 48 h from the moment of inoculation. Cell number in fermentation medium was determined with Neubauer Haemocytometer under 400× magnification using an optical microscope (Wild M20, Heerbrugg, Gais, Switzerland). Viable cells were counted with the methylene blue staining technique (*7*). The free amino nitrogen content was determined by ion-selective electrode. The pH was measured directly in the cultivation medium by the laboratory multiparameter analyzer Consort C863 (Consort, Turnhout, Belgium) with the glass electrode. Total dissolved salt (TDS) content and conductivity were determined using conductivity electrode. The amount of soluble ash was calculated from the following formula (*8*):

$$w(\text{ash})=(0.0018[a-(b\times 0.9)]\times 20)/\%\ \text{dm} \qquad /1/$$

where a is the conductivity of the sample (μS/cm) and b the conductivity of distilled water (μS/cm). Dry matter (dm) was determined by the standard drying method in an oven at 105 °C to a constant mass (*8*).

The samples of the substrates and cultivation media were centrifuged at 4000 rpm for 15 min. Then sucrose and reducing sugar content (sum of glucose and fructose) of the supernatant were determined (Jasco, Inc, Easton, MD, USA, pump PU-980, detector RI-930, sampler AS-950, 20 μL injection loop, column sugar KS-801, eluent: water at flow rate of 0.6 mL/min and elution time 30 min). Fermentable sugar content was expressed as the sum of sucrose and reducing sugars. Sucrose, glucose, and fructose standards were purchased from Supelco (Bellefonte, PA, USA). All chemicals were of reagent grade or better.

Ethanol was determined directly from the samples of the fermentation mash 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 temperature was maintained at 150 °C.

Statistical analyses

All the data presented are the means of three experiments repeated with substrates from two sugar factories. The results were statistically tested by analysis of variance at the significance level of p=0.05. The adequacy of the model was evaluated by coefficient of determination (R^2) and model p-value. Response surface methodology is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out limited number of experiments. For the description of the responses Y (cell number (CFU), ethanol content (% by volume) and fermentable sugar uptake (% by mass), a second-degree polynomial model was fitted to the data:

$$Y=b_{0+}\Sigma b_iX_i+\Sigma b_{ii}{}^2X_{ii}{}^2+\Sigma b_{ij}X_iX_j \qquad /2/$$

where b_0 represents intercept, b_i represents the linear, b_{ii} quadratic and b_{ij} interaction effect of the factors. The factor variables and their ranges are: X_1 fermentation time (0–48 h) and X_2 initial concentration of fermentable sugars in cultivation media based on raw juice (5, 10 and 13 % by mass).

Statistical analyses were performed using Statistica software v. 9.1. (*9*). Response surface plots were generated with the same software and drawn by using the function of two factors.

Results and Discussion

During this experiment three independent cultivations were carried out with raw juices from two different domestic sugar factories as fermentation medium. Results represent average values obtained during those experiments. Analyses of raw materials show that the compositions of raw juices were typical of such intermediate products in domestic sugar factories (Table 1). The observed differences are the consequences of the technological procedure used for sugar beet processing. Given intermediate products with their compositions should be considered as convenient raw materials for the preparation of the cultivation media for bioethanol manufacturing process.

Table 1. Composition of substrates

Raw juice content	SF1	SF2
	w/%	
sucrose	12.85	13.81
reducing sugars	0.07	0.07
fermentable sugars	12.92	13.88
dry matter	14.70	15.80
free amino nitrogen	0.13	0.11
ash content	0.28	0.47

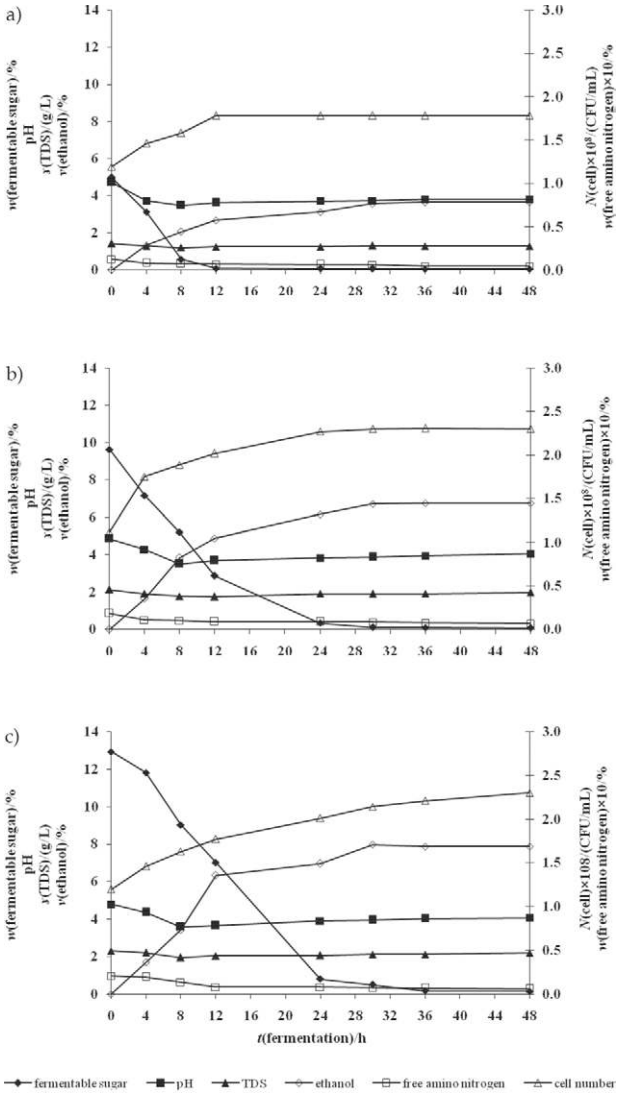


Fig. 1. Ethanol fermentation of culture media based on raw juice with initial fermentable sugar mass fractions of: a) 5, b) 10 and c) 13 %

Course of fermentation of culture media based on raw juice

The course of fermentation of culture media based on raw juice under the applied experimental conditions had to be determined first. This was done by examination of cell number, fermentable sugar, free amino nitrogen, TDS, pH value and ethanol content during fermentation of the media based on raw juice with selected initial fermentable sugar mass fractions. These factors are regularly used in observing the biotechnological process. The results of this procedure can be used for determination of significant factors that may be included in modelling of selected responses.

Figs. 1a-c give the dependence of cell number, fermentable sugar, free amino nitrogen, TDS, pH value and ethanol content during the fermentation of media based on raw juice with initial fermentable sugar mass fractions of 5, 10 and approx. 13 %, respectively. During these experiments, fresh baker's yeast (FB) was used as a production microorganism; this type of yeast is applied in almost all distilleries in our region.

The main metabolic pathway involved in the ethanol fermentation is glycolysis (Embden-Meyerhof-Parnas or EMP pathway), through which one molecule of glucose is metabolized and two molecules of pyruvate are produced. Under anaerobic conditions, the pyruvate is further reduced to ethanol with the release of CO_2. Theoretically, the yield is 0.511 for ethanol and 0.489 for CO_2 on a mass basis of metabolized glucose. Two ATPs produced in the glycolysis are used to drive the biosynthesis of yeast cells, which involves a variety of energy--requiring bioreactions. Therefore, ethanol production is tightly coupled with yeast cell growth, which means that yeast must be produced as a co-product. Without the continuous consumption of ATPs in the growth of yeast cells, the glycolytic metabolism of glucose will be interrupted immediately, because of the intracellular accu-

mulation of ATP, which inhibits phosphofructokinase (PFK), one of the most important regulating enzymes in the glycolysis (*1,10*).

From Fig. 1 it can be seen that during the first 12 h of fermentation yeast cell number increases almost in linear manner in all applied culture media. The exponential phase of yeast cell growth was affected by the residual oxygen content in fermenting mash. After 24 h of fermentation, yeast count reached about $1.78 \cdot 10^8$, $2.27 \cdot 10^8$ and $2.01 \cdot 10^8$ cells/mL in culture media based on initial sugar mass fractions of 5, 10 and approx. 13 %, respectively. With further prolongation of fermentation time, the increase of yeast cell number was insignificant (p=0.6585). These results are in good correlation with literature data shown for sugar beet thick juice and molasses (*11*).

Results shown in Fig. 1 indicate that fermentable sugar content significantly decreased during the fermentation (p=0.0169), coinciding with an increase in biomass and ethanol production. The residual sugar must be controlled at a very low level. For example, no more than 2 and 5 g/L are controlled for residual reducing sugar and total sugar, respectively, in the ethanol production from starch materials. Any ethanol fermentation research, which is expected to be practical, needs to fulfill these criteria (*1*). After 12 h of fermentation of culture media with initial sugar mass fraction of 5 %, the remaining sugar content is negligible. For media with initial sugar mass fraction of 10 and approx. 13 %, residual fermentable sugar content is minimal (approx. 0.5 %) after 24 and 30 h of fermentation, respectively. The presented data are in good correlation with literature data for sugar beet thick juice and molasses (*11*). This fact implies that the cost-effectiveness of longer duration of process should be questioned from the technological and economical point of view.

During fermentation, yeasts assimilate simple compounds, like amino acids and ammonium solids, and generate complexes. According to the obtained results (Fig. 1), during the first 12 h of fermentation nitrogen concentration significantly decreased in all fermenting mashes (p=0.0218). During the exponential phase of yeast cell growth, assimilated nitrogen was incorporated into biomass. With further prolongation of the fermentation time, the nitrogen content slowly decreased for all applied mashes and this decrease was not as significant as in the first 12 h. Total decrease of nitrogen content during 48 h of fermentation in all applied mashes was 0.04–0.07 % (by mass).

Ethanol production is closely related to the growth of yeast cells. The ethanol fermentation rate of non-growing yeast cells is at least 30-fold slower than that of the growing ones (*12*). According to the obtained results (Fig. 1), ethanol content in culture media during the first 12 h of fermentation of mashes with all applied initial sugar mass fractions significantly increased. During further fermentation up to 48 h, ethanol content in mash with sugar mass fraction of 5 % was slightly changed, corresponding to minor remaining sugar content and stagnation of yeast cell growth. With initial fermentable sugar mass fractions of 10 and 13 %, intensive increase of ethanol volume fraction was prolonged until 30 h. During further fermentation time, ethanol volume fractions were not significantly changed (p=0.1401). The obtained ethanol content at the end of fermentation of media based on raw juice with initial fermentable sugar mass fractions of 5, 10 and 13 % was 3.64, 6.75 and 7.88 % (by volume), respectively. These results indicate that during raw juice fermentation, the obtained ethanol content is similar to the content obtained during thick juice and molasses fermentation (*11*). This fact implies that lower purity of raw juice does not have a negative impact on ethanol production.

pH value and TDS content of all applied culture media were slowly decreased during the first 12 h of fermentation and afterwards remained almost unchanged. During growth, it is important for the yeast to preserve a constant intracellular pH. There are many enzymes functioning within the yeast cell during growth and metabolism. Each enzyme works best at its optimal pH, which is acidic because of the acidophilic nature of the yeast itself. When the extracellular pH deviates from the optimal level, the yeast cells need to invest the energy to either pump in or pump out hydrogen ions in order to maintain the optimal intracellular pH. If the extracellular pH deviates too much from the optimal range, it may become too difficult for the cell to maintain constant intracellular pH, and the enzymes may not function normally. If the enzymes are deactivated, the yeast cell will not be able to grow and produce ethanol efficiently (*13*). According to the results in Fig. 1, pH values remained in the optimal range for yeast cell activities during total fermentation time of culture media based on raw juice with initial fermentable sugar mass fractions of 5, 10 and 13 %. It can be concluded that under the experimental conditions applied during this research, correction of this parameter at some stage of cultivation is not necessary.

Statistical analyses of the modelled responses

The results of the statistical analyses are presented in Table 2. The coefficients are related to actual variables. The ANOVA results for modelled responses are reported in Table 3. Relatively high values of R^2, obtained for all responses, indicate good fit of the experimental data to Eq. 2. All polynomial models tested for the selected responses were significant at 95 % confidence level (p= 0.05, Table 3). The model F-values of 46.62, 90.30 and 99.39 for cell number, ethanol content and fermentable sugar uptake, respectively, imply that models for selected responses are significant. Fig. 2 shows the parity plot of the observed and predicted values for modelled responses.

Mathematical model of raw juice fermentation

The goodness of fit of the model was checked by the determination coefficient that was found to be 0.928 for the response of cell number, which indicates that less than 10 % of the variations could not be explained by the model. As for significance of the polynomial coefficients, their p-values suggest that the most important factor influencing cell number is initial mass fraction of fermentable sugars. The mutual interaction between ini-

Table 2. Regression equation coefficients for selected responses

Effects	Cell number		Ethanol volume fraction		Fermentable sugars	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
intercept						
b_0	0.299	0.23992	–2.030	0.1324	1.142	0.4775
linear						
b_1	0.270	0.0002	0.722	0.0261	0.426	0.3734
b_2	0.031	0.0002	0.155	0.0010	–0.255	0.0001
quadratic						
b_{11}	–0.015	0.00017	–0.038	0.0341	0.036	0.1790
b_{22}	–0.001	<0.0001	–0.004	<0.0001	0.007	<0.0001
interaction						
b_{12}	0.002	0.0018	0.013	<0.0001	–0.027	<0.0001

Table 3. Analysis of variance (ANOVA) of the modelled responses

Response	Source						F-value	p-value	R^2
	Residual			Model					
	DF	SS	MS	DF	SS	MS			
cell number	18	0.22	0.012	5	2.89	0.58	46.62	<0.0001	0.928
ethanol volume fraction	18	5.74	0.32	5	143.96	28.79	90.30	<0.0001	0.962
fermentable sugars	18	14.20	0.79	5	391.95	78.39	99.39	<0.0001	0.965

DF – degree of freedom, SS – sum of squares, MS – mean squares

tial mass fraction of fermentable sugars and fermentation time is significant at the level 0.05, shown in Fig. 3.

For the response of ethanol volume fraction, coefficient of determination was found to be 0.962, which indicates that only 3.8 % of the variations could not be explained by the model. Fermentation time is less significant compared to the initial mass fraction of fermentable sugars for linear as well as quadratic effects. The effects of initial mass fraction of fermentable sugars and fermentation time on ethanol volume fraction are presented in Fig. 4.

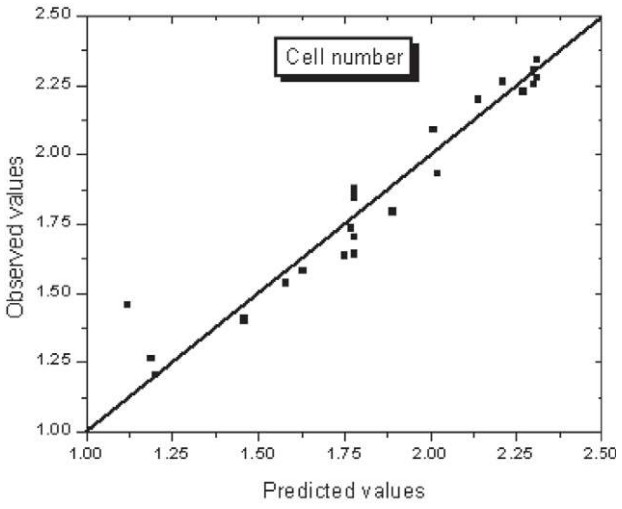
The goodness of fit of the model was checked by the determination coefficient, which was found to be 0.965 for the response of fermentable sugar, indicating that less than 5 % of the variations could not be explained by the model. As for significance of the polynomial coefficients, their p-values suggest that the most important linear factor is initial concentration of fermentable sugars and the same conclusion can be applied to quadratic effects.

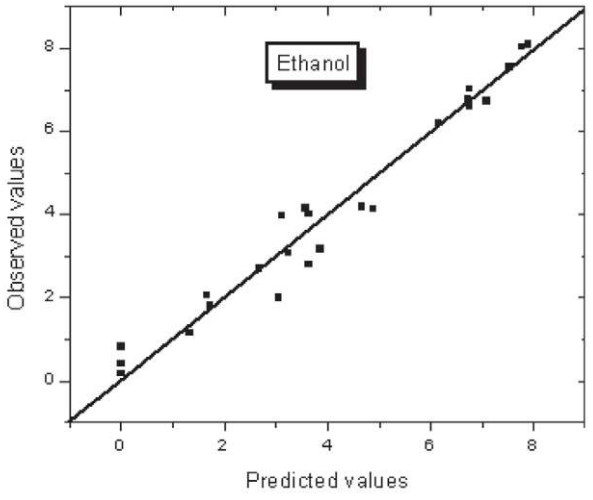
The effects of initial mass fraction of fermentable sugars on sugar uptake during fermentation time are shown in Fig. 5.

Optimization of raw juice fermentation

The final goal of response surface methodology is the process optimization. Thus, the developed models can be used for simulation and optimization. To optimize the process with two or more output responses, it is helpful to use the concept of desirability function. The desirability function is one of the most widely used methods for optimization of multiple response processes in science and engineering (*14*). It combines multiple responses into one response called desirability function by choice of value from 0 (one or more characteristics are unacceptable) to 1 (all process characteristics are on target). Each of the estimated responses is transformed to an individual desirability value ranging from 0 to 1. The value of individual desirability increases as the desirability of the corresponding response increases. The overall desirability of the process is computed as geometric mean of the individual desirability functions (*15*).

The results of optimization by desirability function approach for maximization of cell number and ethanol volume fraction are given in Fig. 6. These conditions were selected taking into account that the ethanol fermentation rate of growing yeast cells is considerably faster than that of the non-growing ones. As it can be seen from Fig. 6, the highest values of desirability function are obtained in the region of high initial sugar mass fractions and long fermentation times. The optimal values of fermentation time and initial sugar mass fraction are 38 h and 12.30 % (by mass), respectively, for the highest value of desirability function *i.e.* 1. Even so, the fermentation time needed to reach desirability around 0.9 varies from 30 to 48 h, which is in good agreement with the industrial practice. On the other hand, initial sugar mass fraction for the same region of desirability function varies in the region between 10 % for fermentation time of 40 h and 13 % for fermentation time of around 30 h. To select optimal fermentation time and initial sugar mass fraction for industrial application, further techno-economic analysis should be done.





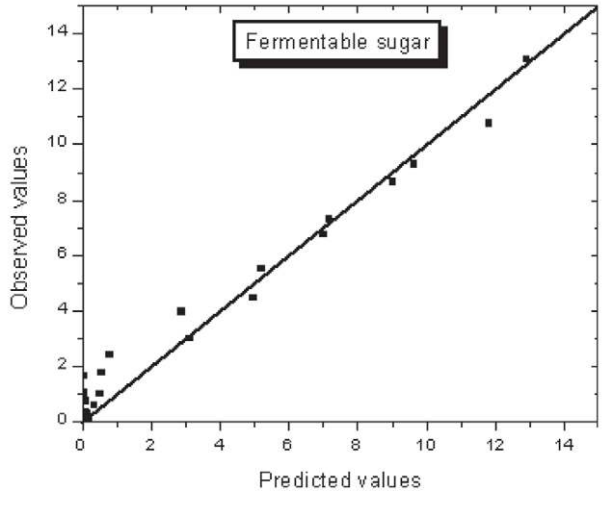


Fig. 2. Parity plot showing observed *vs.* predicted values for modelled responses

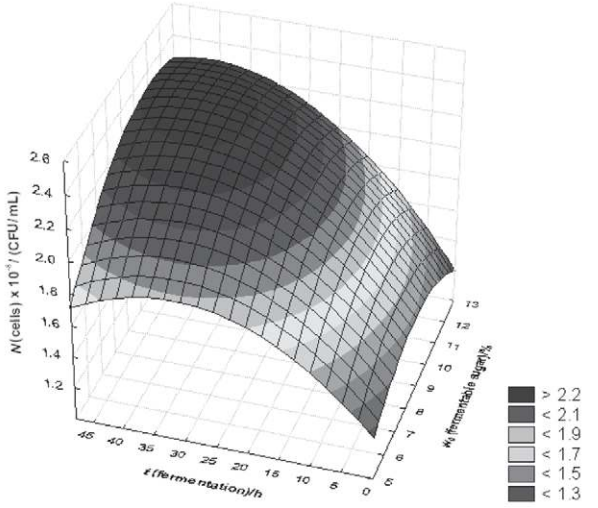


Fig. 3. The effects of initial mass fraction (w_0) of fermentable sugars and fermentation time on the cell number

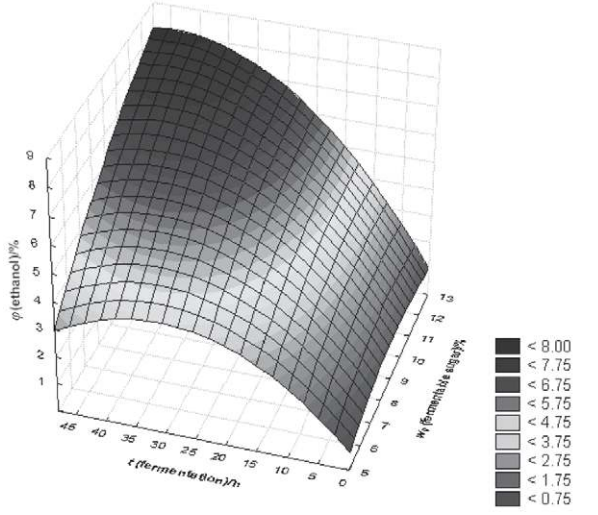


Fig. 4. The effects of initial mass fraction (w_0) of fermentable sugars and fermentation time on ethanol volume fraction

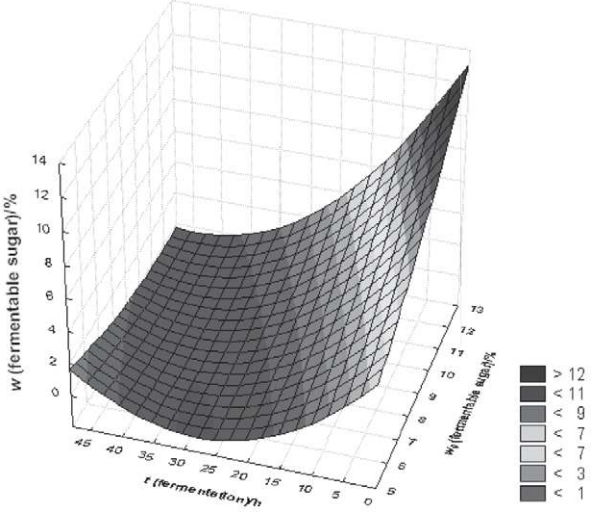


Fig. 5. The effects of initial mass fraction (w_0) of fermentable sugars and fermentation time on fermentable sugar

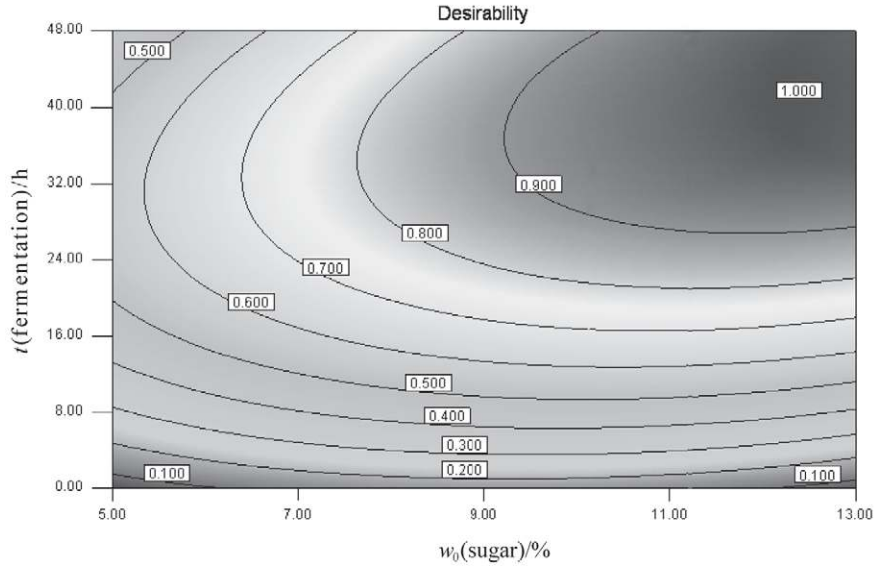


Fig. 6. The overall desirability function of the fermentation process

Productivity of different yeast types

Culture media, prepared from raw juice with the degree of dilution adjusted to achieve initial mass fraction of fermentable sugars estimated as optimal, were inoculated with different types of yeast in order to evaluate their fermentation productivity. Examined types of yeast are commercially available and extensively used as starter cultures in various branches of the fermentation industry. Dried distiller's yeast (DD) is planned for use in ethanol fuel and alcohol fermentations for beverages. Yeasts DW1 and DW2 are used for wine production and are selected because of very short lag phase and rapid fermentation. Strain DW2 is also tolerant of high ethanol volume fractions. The same strain of commercially produced baker's yeast is applied in two different forms, as fresh and dried yeast (DB and FB).

Fig. 7 shows the dependence of the ethanol production on the fermentation time and the applied yeast strains. In the first 4 hours of fermentation, productivity of FB was considerably higher in comparison with other types of yeast applied in dried forms, DD, DW1, DW2 and DB. This fact can be explained with diverse lengths of lag phases for different types of yeast. The length of the lag phase depends on factors such as nutrition, growth conditions, inoculation density, temperature and growth history of the inoculum (*12*). According to the statistical analyses of illustrated results (Fig. 4), ethanol production was significantly increased during the first 12 h of fermentation for all applied yeast strains, DD, DW1, DW2, DB and FB ($p=0.0008$). During this period, maximum production of 2.10, 2.07, 2.28, 2.37 and 2.19 g/(L·h) for DD, DW1, DW2, DB and FB, respectively, was achieved. In the further course of fermentation up to 48 h, the productivity significantly decreased (Fig. 7) for all examined yeast strains ($p<0.0001$). Experimental results (Fig. 7) indicate that there is no statistical difference between productivity of the applied yeast strains

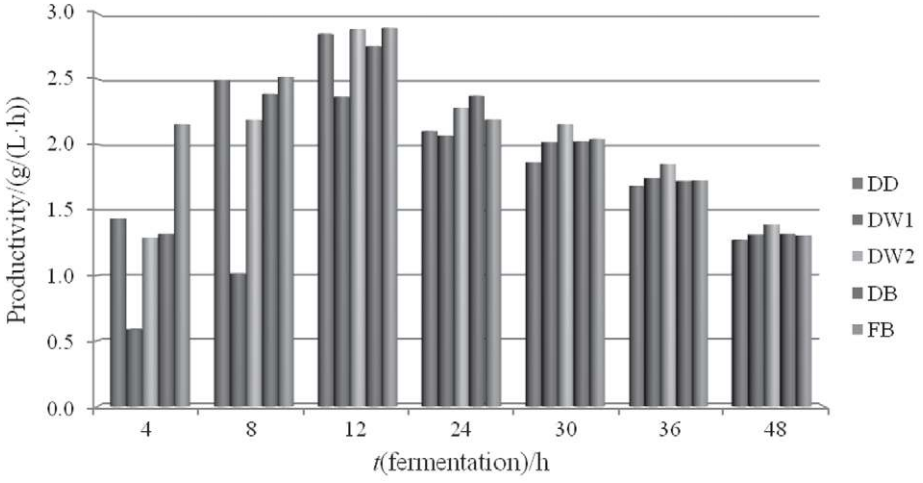


Fig. 7. Dependence of the ethanol production on different yeast strains during fermentation of raw juice (DD – dried distiller's yeast, DW1 – dried wine yeast, DW2 – dried wine yeast tolerant of high ethanol volume fractions, DB – dried baker's yeast and FB – fresh baker's yeast)

after 12 h of fermentation. This fact implies that further analyses and optimisation for selecting optimal fermentation time and initial sugar mass fraction for industrial application can be done with one of the examined strains, meaning that the obtained responses will be valid for each of the five strains applied during this research, DD, DW1, DW2, DB and FB.

Conclusion

This research has confirmed that efficient ethanol production from raw juice as intermediate product of sugar beet processing is technically possible with all examined commercial strains of yeast *Saccharomyces cerevisiae*. Under the applied experimental conditions, raw juice can be used for fermentation mash preparation without any addition of nutrients, with only initial pH correction. The regression equations obtained in this study can be used to find the optimum conditions of fermentation process in industrial scale from economic point of view. Extrapolation of the responses beyond the range of used experimental values may not be valid.

Acknowledgement

Financial support from the Ministry of Science of the Republic of Serbia is highly acknowledged (grant BTN-20009).

References

1. F.W. Bai, W.A. Anderson, M. Moo-Young, Ethanol fermentation technologies from sugar and starch feedstocks, *Biotechnol. Adv. 26* (2008) 89–105.
2. S.N. Dodić, S.D. Popov, J.M. Dodić, J.A. Ranković, Z.Z. Zavargo, Potential contribution of bioethanol fuel to the transport sector of Vojvodina, *Renew. Sust. Energ. Rev. 13* (2009) 2197–2200.
3. S.N. Dodić, S.D. Popov, J.M. Dodić, J.A. Ranković, Z.Z. Zavargo, Potential development of bioethanol production in Vojvodina, *Renew. Sust. Energ. Rev. 13* (2009) 2722–2727.
4. S.N. Dodić, S.D. Popov, J.M. Dodić, J.A. Ranković, Z.Z. Zavargo, Biomass energy in Vojvodina: Market conditions, environment and food security, *Renew. Sust. Energ. Rev. 14* (2009) 862–867.
5. A. Hinková, Z. Bubník, Sugar beet as a raw material for bioethanol production, *Czech. J. Food Sci. 19* (2001) 224–234.
6. S. Henke, Z. Bubník, A. Hinková, V. Pour, Model of sugar factory with bioethanol production in program Sugars™, *J. Food Eng. 77* (2006) 416–420.
7. V.R. McDonald, Direct microscopic technique to detect viable yeast cells in pasteurized orange drink, *J. Food Sci. 28* (1963) 135–139.
8. Official Methods of Analysis, AOAC International, Gaithersburg, MD, USA (2000).
9. Statistica (Data Analyses Software System) v. 9.1, StatSoft, Inc, Tulsa, OK, USA (2010) (*www.statsoft.com).*
10. N. Kosaric, F. Vardar-Sukan: Potential Source of Energy and Chemical Products. In: *The Biotechnology of Ethanol, Classical and Future Applications*, M. Roehr (Ed.), Wiley-VCH, Weinheim, Germany (2001) pp. 89–106.
11. S. Dodić, S. Popov, J. Dodić, J. Ranković, Z. Zavargo, R. Jevtić Mučibabić, Bioethanol production from thick juice as intermediate of sugar beet processing, *Biomass Bioenergy, 33* (2009) 822–827.
12. W.M. Ingledew: Alcohol Production by *Saccharomyces cerevisiae:* A Yeast Primer. In: *The Alcohol Textbook*, Nottingham University Press, Nottingham, UK (1999).
13. N.V. Narendranath, K.C. Thomas, W.M. Ingledew, Acetic acid and lactic acid inhibition of growth of *Saccharomyces cerevisiae* by different mechanisms, *J. Am. Soc. Brew. Chem. 59* (2001) 187–194.
14. M. Khayet, C. Cojocaru, G. Zakrzewska-Trznadel, Response surface modelling and optimization in pervaporation, *J. Membr. Sci. 321* (2008) 272–283.
15. C. Cojocaru, M. Khayet, G. Zakrzewska-Trznadel, A. Jaworska, Modeling and multi-response optimization of pervaporation of organic aqueous solutions using desirability function approach, *J. Hazard. Mater. 167* (2009) 52–63.