Reza Shaghaghi-Moghaddam, Hoda Jafarizadeh-Malmiri*, Parviz Mehdikhani, Reza Alijanianzadeh and Sepide Jalalian

Optimization of submerged fermentation conditions to overproduce bioethanol using two industrial and traditional *Saccharomyces cerevisiae* strains

https://doi.org/10.1515/gps-2018-0044
Received February 7, 2018; accepted February 20, 2018; previously published online April 12, 2018

Abstract: The present study focuses on the overproduction of bioethanol through submerged fermentation. In a batch-scale submerged bioreactor using a traditional and an industrial *Saccharomyces cerevisiae* (NCYC 4109 and SFO6) strains, the fermentation was accomplished. The effects of the substrate brix (20.50–24.00 °Bx) and inoculum percentage in the initial fermentation solution (15%–45%) as independent variables on bioethanol production (g/l) as the dependent variable were assessed using the response surface methodology. Using the obtained experimental values for the response variable based on experiments for the fermentation parameters, a general model (second-order) with high coefficient of determination values ($R^2 > 95\%$) was generated to predict the bioethanol concentrations that were obtained using both yeast strains. The obtained results indicated that the optimum fermentation conditions to overproduce bioethanol (56.14 g/l) using the SFO6 yeast were at the substrate brix and inoculum percentage values of 24.70 °Bx and 26.35%, respectively. However, a higher concentration of bioethanol (53.1 g/l) using the NCYC 4109 yeast strain was obtained at the substrate brix and inoculum percentage values of 24.68 °Bx and 40.07%, respectively.

Keywords: bioethanol production; inoculum density; optimization; response surface methodology; *Saccharomyces cerevisiae*.

***Corresponding author: Hoda Jafarizadeh-Malmiri,** Faculty of Chemical Engineering, Sahand University of Technology, East Azarbaijan 51335-1996, Tabriz, Iran, e-mail: h_jafarizadeh@sut.ac.ir
Reza Shaghaghi-Moghaddam: Faculty of Chemical Engineering, Sahand University of Technology, East Azarbaijan 51335-1996, Tabriz, Iran
Parviz Mehdikhani: Agricultural Researches and Education Natural Resources Center, West Azarbaijan, Urmia, Iran
Reza Alijanianzadeh and Sepide Jalalian: Research and Development Center, Bidestan Company, Qazvin, Iran

1 Introduction

After the overutilization of fossil fuels in the past, concerns are being raised regarding their exhaustible nature and their negative environmental impacts [1]. In the last decades, there have been many attempts to develop new processes for finding replacements that do not have their negative features but are commercially practical. Bioethanol and biodiesel can be produced from agricultural products and wastes, and have attracted attention because of their huge and diverse raw material availability [2–5]. Compared to the conventional fossil fuels, bioethanol has numerous advantages such as lower emission of volatile organic compounds and higher specific energy and heat of vaporization [6]. Bioethanol, as with other sustainable energy sources, has got its own advantages and disadvantages. One of the disadvantages of the current production routes is the low productivity of the process in comparison with the current chemical process, which has motivated the manipulation of the process conditions in order to increase productivity [7]. Studies have shown that the performance of fermentation is highly dependent on the type of microorganism strain that is used as the producing agent, the type and volume of the bioreactors, the type and composition of culture media and proper nutrients, and the pH and temperature of the fermentation media [8–10].

One of the factors that can significantly affect the fermentation efficiency is the initial concentration of the activated yeast in the medium, which should be able to tolerate the higher osmotic pressure of the growth medium and bioethanol concentration [11, 12]. As the initial concentration of the inoculum is increased, the required time for the completion of the fermentation process would decrease, and consequently the amount of alcohol produced will also decrease because of the lesser quantity of substrate present in the medium. Thus, there should be a balance between the speed of production of bioethanol and the appropriate final concentration [10, 13–15].

Some studies have arrived at appropriate models for describing the produced bioethanol based on process variables such as temperature, substrate type, and

Open Access. © 2019 Walter de Gruyter GmbH, Berlin/Boston

concentration. Laluce et al. utilized the response surface methodology (RSM) with central composite design (CCD) as a statistical analysis and design procedure in order to better monitor, model, and optimize the inoculum size, substrate density, and temperature in bioethanol production. Their study revealed such independent variables' effects on bioethanol production and also showed that statistical procedures could be successfully utilized for modeling to optimize the process [15]. In fact, statistical methods can easily determine the interaction effects of the independent parameters on the responses [16]. On the other hand, the obtained mathematical models generated using experimental data can be used to predict the responses of the process and to find the optimum process conditions [10].

In this work, an industrial and a traditional *Saccharomyces cerevisiae* strains, which were screened in our previous study, were used to overproduce bioethanol selected through optimization [17]. In order to do that, the substrate brix (°Bx) and the inoculum percentage were used as independent variables and alcohol production (g/l) as the dependent variable. Experimental design and RSM were used to evaluate the effects of changing the variables on the rapid fermentation in bioethanol production.

2 Materials and methods

2.1 Materials

The molasses used as the substrate was supplied by Sahand Sugar Beet Company (Khoy, Iran). It has a brix value, total amount of reduced sugar, pH, ash content, and density of 74.07 (°Bx), 48.65%, 6.16, 9.6% (v/v), and 1.385×10^3 (kg/m^3), respectively. Based on the obtained results of our previous study, two different kinds of dried form of *S. cerevisiae* strains, namely NCYC 4109 (a traditional bakery yeast from the National Collection of Yeast Cultures, Norwich, UK) and SFO6 (an industrial yeast from Iran Mayeh Company, Tehran, Iran), were selected and provided by the Agricultural Research and Education Natural Resources Center (West Azerbaijan, Urmia, Iran) [17]. Sulfuric acid, diammonium hydrogen phosphate (DHP), and urea as the pH adjuster, P, and N sources, respectively, were purchased from the Dr. Mojallali Company (Tehran, Iran).

2.2 Inoculum preparation and anaerobic fermentation

In order to prepare of the inoculum (biomass), sterile molasses was diluted using distilled water to provide a diluted molasses solution with a brix value of 11.24 (°Bx), and its pH was set to 4.2 using sulfuric acid (30% v/v). Urea (250 ppm) and DHP (500 ppm) were added to the substrate to increase its nutritional value. Each provided dried yeast (0.3 g) was added to 1 l of the prepared substrate. The samples were incubated at 32°C for 14 h and then aerated (1 volume of air per volume of liquid per minute).

In order to produce bioethanol, batches of submerged fermentation molasses with different brix values (20.5–24.00 °Bx) were prepared and used as the fermentation substrates. They were added to different amounts of the provided inoculum (15–30 ml) to obtain 100 ml each of the fermented solution (including the substrate and inoculum). The solution mixtures were then poured into 250-ml sealed bottles and incubated at 32°C for 32 h.

2.3 Analysis

Concentration of bioethanol: At the end of anaerobic fermentation, the concentration of the produced bioethanol was calculated using the technique described by Son et al. [18]. In this method, after bioethanol distillation, the true brix value in the distilled fermentation broth was measured by a refractometer (Index instrument Ltd., Kissimmee, FL, USA) and the bioethanol concentration using a hydrometer.

2.4 Design of experiments and statistical analysis

As a useful statistical method, RSM has numerous advantages including designing experiments that yield sufficient and consistent measurements of the studied responses, achieving a model that best fits the experimental data, and gaining optimum values of the experimental parameters that lead to target values for the responses [16, 19–21].

According to the literature, the effects of two independent variables, namely the substrate brix (°Bx, X_1) and percentage of inoculum (%, X_2), in the initial fermentation solutions on the response variable, namely the bioethanol concentration (g/l, Y), were evaluated using RSM [15, 17]. The experiments were designed by a two-factor CCD based on cubic points and using one block, which indicated that the total experiment runs should be 13 with five replicates for the center points [16]. Table 1 shows the levels of the studied variables. A general polynomial model was used to establish the relationship between the response variable of the fermentation process and the two independent variables for each microorganism:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

where Y and X_i are related to the bioethanol concentration and fermentation parameters, respectively. Furthermore, β is a constant, and β_i, β_{ii}, and β_{ij} represent the coefficients of the main, quadratic, and interaction terms, respectively. Significant differences between the fermentation parameters were analyzed using the analysis of variance (ANOVA) and based on the p-value. In fact, a small p-value ($p < 0.05$) means a significant effect of the terms (e.g. linear,

Table 1: Fermentation conditions and their levels.

Independent variables	Unit	Levels		
		Low	Middle	High
X_1, substrate brix	(°Bx)	20.50	22.25	24.00
X_2, inoculum percentage	(%)	15	30	45

quadratic, and interaction) on the responses [22]. Adequacy of the generated models was evaluated using the coefficient of determination (R^2 and R^2-adjusted) [20].

2.5 Optimization procedure and model verification

To better visualize the interaction effects of the fermentation variables on the bioethanol concentration, three-dimensional surface plots were established for each yeast strain [14]. In order to show graphically the optimum area, the contour plots were used [19]. For obtaining the exact values of the optimized fermentation conditions under which bioethanol with highest concentration was produced, numerical optimization was used [23]. To verify the acceptability of the fitted models, Tukey's comparison test was performed between the values of the predicted and experimental bioethanol concentration at the obtained optimum fermentation conditions. The Minitab statistical software (Minitab Inc., version 16.2.4, PA, USA) was used for CCD, RSM, ANOVA, Tukey's test, optimization, and verification procedures.

Table 3: Matrix of the CCD for bioethanol production using the NCYC 4109 yeast.

Run	Substrate brix (°Bx)	Inoculum (%)	Bioethanol concentration (g/l)[a]	Bioethanol concentration (g/l)[b]
1	24.72	30.0	50.34	50.14
2	22.25	30.0	51.68	51.05
3	20.50	15.0	44.97	45.07
4	24.00	45.0	51.29	51.26
5	22.25	30.0	52.07	51.05
6	22.25	30.0	52.47	51.05
7	22.25	30.0	51.68	51.05
8	22.25	30.0	50.89	51.05
9	20.50	45.0	41.20	38.23
10	19.78	30.0	44.97	45.95
11	22.25	51.2	39.45	40.51
12	24.00	15.0	35.40	37.96
13	22.25	8.8	37.08	35.94

[a]Experimental value.
[b]Predicted value.
CCD, Central composite design.

3 Results and discussion

3.1 Response surface models investigation

The response values for the experimental data (Tables 2 and 3) were used to fit and generate second-order polynomial models for studying bioethanol production as a function of the fermentation variables using the SFO6 and NCYC 4109 yeast strains, respectively. The estimated regression coefficients and p-values of all terms are given in Tables 4 and 5 for the fitted models based on SFO6 and NCYC 4109 strains, respectively. As can be seen from Table 4, among all the terms, the main term (i.e. the inoculum percentage) in the fermentation solution and quadratic term (i.e. substrate brix) had only insignificant effects on the bioethanol production using the industrial yeast strain (SFO6). The high value (0.9653) of the coefficient of determination (R^2) and the reasonably high value (0.7622) of R^2-adjusted confirmed the suitability of the resulting model for predicting the produced bioethanol concentration using the yeast SFO6 within the defined ranges for

Table 2: Matrix of the CCD for bioethanol production using the SFO6 yeast.

Run	Substrate brix (°Bx)	Inoculum (%)	Bioethanol concentration (g/l)[a]	Bioethanol concentration (g/l)[b]
1	24.72	30.0	55.62	55.91
2	22.25	30.0	55.23	55.27
3	20.50	15.0	49.70	49.27
4	24.00	45.0	50.49	50.92
5	22.25	30.0	55.23	55.27
6	22.25	30.0	54.99	55.27
7	22.25	30.0	55.38	55.27
8	22.25	30.0	55.54	55.27
9	20.50	45.0	50.6	51.43
10	19.78	30.0	53.49	53.20
11	22.25	51.2	48.78	47.88
12	24.00	15.0	54.44	53.60
13	22.25	8.8	47.34	48.24

[a]Experimental value.
[b]Predicted value.
CCD, Central composite design.

Table 4: p-Values and regression coefficients for the generated model based on the yeast strain SFO6.

p-Value			Regression coefficient	
Parameters	Independent variables	p-Value	β	Coefficient
Constant		0.000	β_0 (constant)	55.27
Main	X_1	0.008	β_1	0.95
	X_2	0.638	β_2	−0.12
Quadratic	X_1^2	0.236	β_{11}	−0.35
	X_2^2	0.000	β_{22}	−3.60
Interaction	X_1X_2	0.013	β_{12}	−1.20
R^2		0.9653		
R^2-adjusted		0.7622		

1, Substrate brix (°Bx); 2, inoculum (%).

Table 5: p-Values and regression coefficients for the generated model based on the yeast strain NCYC 4109.

p-Value			Regression coefficient	
Parameters	**Independent variables**	**p-Value**	β	**Coefficient**
Constant		0.000	β_0 (constant)	51.05
Main	X_1	0.010	β_1	1.48
	X_2	0.006	β_2	1.61
Quadratic	X_1^2	0.012	β_{11}	−1.50
	X_2^2	0.000	β_{22}	−6.41
Interaction	X_1X_2	0.000	β_{12}	5.03
R^2		0.9776		
R^2-adjusted		0.8508		

1, Substrate brix (°Bx); 2, inoculum (%).

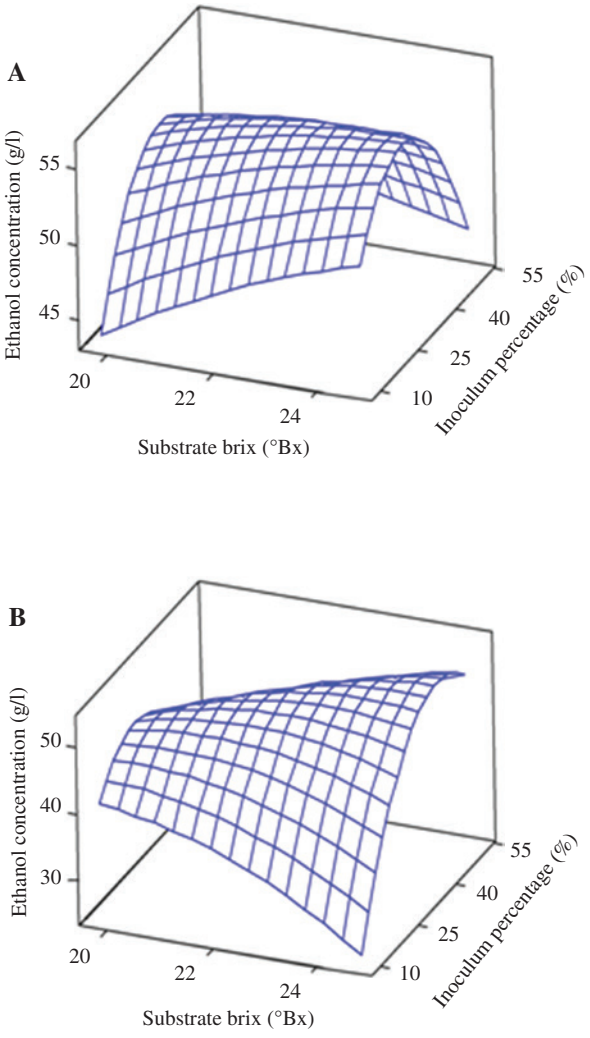

Figure 1: Surface plots of the concentration of the produced bioethanol (g/l) as function of the substrate brix (°Bx) and inoculum percentage (%), during fermentation using the yeast strains (A) SFO6 and (B) NCYC 4109.

the fermentation conditions. As can be seen from Table 5, all the terms in the generated model for predicting bioethanol concentration throughout the fermentation process using a traditional bakery yeast (NCYC 4109) had significant ($p < 0.05$) effects. The higher values of R^2 (0.9776) and R^2-adjusted (0.8508) also verified the fitness of the proposed model.

3.2 Influence of the fermentation conditions on the bioethanol concentration

Effects of the fermentation parameters, namely substrate brix and percentage of inoculum in the initial fermentation solution, on the concentration of the produced bioethanol using SFO6 and NCYC 4109 yeast strains are presented in Figure 1A and B, respectively. As can be seen in Figure 1A, during bioethanol production using the yeast strain SFO6, at a lower substrate brix, by increasing the inoculum percentage the concentration of produced bioethanol increased. However, at higher substrate brix, with an increase in the amount of inoculum, the concentration of the produced bioethanol increased and then decreased. This can be described by the fact that at low substrate brix, a high amount of inoculum can drastically decrease the duration of the yeast lag phase of growth, which in turn could initiate the bioethanol production. On the other hand, at high substrate brix, it seems that by increasing the percentage of inoculum, the concentration of biomass and bioethanol increased, which in turn increased the osmotic pressure of the fermentation broth and inhibited the growth of the yeast. Therefore, the bioethanol production decreased, as can be seen at higher substrate brix and inoculum percentage (Figure 1A). As can be seen in Table 2, the concentration of bioethanol produced by the yeast SFO6 changed from 47.34 to 55.62 g/l, while concentration of the produced bioethanol using the yeast NCYC 4109 varied from 35.40 to 52.47 g/l (Table 3). As can be seen in Figure 1B, at higher substrate brix, by increasing the percentage of inoculum (NCYC 4109) in the initial fermentation solution, the concentration of the produced bioethanol increased. The opposite trends in the produced bioethanol concentration using both yeasts at higher substrate brix and the amount of inoculum revealed that the traditional bakery yeast (NCYC 4109) was tolerant to the high osmotic pressure of the fermentation broth. However, the concentration of the produced bioethanol using this yeast was lower than that produced using the industrial yeast (SFO6), as can be observed in Tables 2 and 3.

3.3 Optimization of the submerged fermentation conditions to overproduce bioethanol

Graphical optimizations of the fermentation conditions using the yeast strains SFO6 and NCYC 4109 to overproduce bioethanol are indicated in Figure 2A and B, respectively. As can be seen in Figure 2A, the minimum concentrations for the produced bioethanol using the industrial yeast (SFO6) were obtained at lower substrate brix and inoculum percentage and higher substrate brix and percentage of inoculum. However, the maximum concentrations of the produced bioethanol using the yeast of SFO6 were obtained at the substrate brix ranging from 19 to 35 (°Bx) and an inoculum percentage value of higher than 22%. Numerical optimization indicated that the maximum concentration of the produced bioethanol using SFO6 (56.14 g/l) was obtained at the substrate brix and inoculum percentage values of 24.70 °Bx and 26.35%, respectively. As can be seen in Figure 2B, the minimum concentrations for the produced bioethanol using the traditional bakery yeast (NCYC 4109) were obtained at lower substrate brix and higher inoculum percentage and at lower inoculum percentage and higher substrate brix, while higher concentrations of the produced bioethanol, using the yeast of NCYC 4109, were obtained at both higher substrate brix (>21 °Bx) and inoculum percentage (>25%). Numerical optimization illustrated that the maximum concentration of the produced bioethanol using NCYC 4109 (53.1 g/l) was obtained at the substrate brix and inoculum percentage values of 24.68 °Bx and 40.07%, respectively. The nonsignificant differences between the values of the experimental and predicted concentration of the produced bioethanol at the obtained optimum fermentation conditions using the yeasts strains SFO6 and NCYC 4109 verified the adequacy of the generated models. The experimental values for the concentrations of the produced bioethanol at the obtained optimum conditions using SFO6 and NCYC 4109 were 55.2 ± 2 and 54.23 ± 2 g/l, respectively.

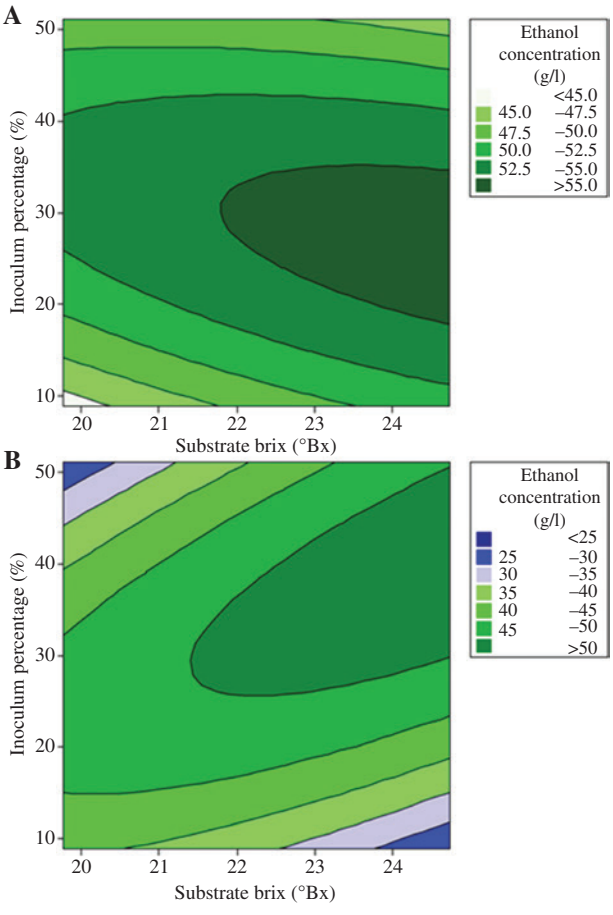

Figure 2: Contour plots for concentration of the produced bioethanol (g/l) as function of the substrate brix (°Bx) and inoculum percentage (%), during fermentation using the yeast strains (A) SFO6 and (B) NCYC 4109.

4 Conclusions

Based on the above study, it can be concluded that RSM can be an effective method to predict, model, and optimize bioethanol production by manipulating the process variables. In this regard, the influence of the interaction of the substrate brix and the inoculum percentage was remarkable on bioethanol production for two selected (industrial and traditional bakery) yeast strains in order to maximize the productivity. However, the findings of the present study revealed that the industrial strain had higher resistance against osmotic pressure of the fermented broth than the traditional bakery yeast strain.

Acknowledgments: The authors would like to thank Bidestan Company for analysis and material support.

Conflict of interest statement: The authors declare no conflict of interest.

References

[1] Hill J, Nelson E, Tilman D, Polasky S, Tiffany D. *Proc. Natl. Acad. Sci.* 2006, 103, 11206–11210.
[2] Crutzen PJ, Mosier AR, Smith KA, Winiwarter W. *Atmos. Chem. Phys.* 2008, 8, 389–395.
[3] Sadik MW, Asmaa A. *Int. J. Curr. Microbiol. Appl. Sci.* 2014, 3, 804–818.

[4] Martínez O, Sánchez A, Font X, Barrena R. *J. Clean. Prod.* 2017, 158, 8–17.
[5] Mayzuhroh A, Arindhani S, Caroenchai C. *Agric. Agric. Sci. Procedia* 2016, 9, 493–499.
[6] Meenakshi A, Kumaresan R. *Int. J. Chemtech. Res.* 2014, 6, 2843–2853.
[7] Mehdikhani P, Bari MR, Hovsepyan H. *Afr. J. Microbiol. Res.* 2011, 5, 2654–2660.
[8] Lin Y, Zhang W, Li C, Sakakibara K, Tanaka S, Kong H. *Biomass Bioenergy* 2012, 47, 395–401.
[9] Takeshige K, Ouchi K. *J. Ferment. Bioeng.* 1995, 79, 449–452.
[10] Betiku E, Taiwo AE. *Renew. Energy* 2015, 74, 87–94.
[11] Ergun M, Mutlu SF. *Bioresour. Technol.* 2000, 73, 251–255.
[12] Chandel AK, Narasu ML, Chandrasekhar G, Manikyam A, Rao LV. *Bioresour. Technol.* 2009, 100, 2404–2410.
[13] Le Man H, Behera S, Park H. *Int. J. Environ. Sci. Technol.* 2010, 7, 157–164.
[14] Maiti B, Rathore A, Srivastava S, Shekhawat M, Srivastava P. *Appl. Microbiol. Biotechnol.* 2011, 90, 385–395.
[15] Laluce C, Tognolli JO, De Oliveira KF, Souza CS, Morais MR. *Appl. Microbiol. Biotechnol.* 2009, 83, 627–637.
[16] Jafari N, Jafarizadeh-Malmiri H, Hamzeh-Mivehroud M, Adibpour M. *Green Process. Synth.* 2017, 6, 333–340.
[17] Shaghaghi-Moghadam R, Jafarizadeh-Malmiri H, Mehdikhani P, Jalalian S, Alijanianzadeh R. *Z. Naturforsch.* 2018, 73, 361–366.
[18] Son H, Hong Y, Park W, Yu M, Lee C. *J. Food Sci.* 2009, 74, 106–111.
[19] Eskandari-Nojehdehi M, Jafarizadeh-Malmiri H, Rahbar-Shahrouzi J. *Nanotechnol. Rev.* 2016, 5, 537–548.
[20] Mohammadlou M, Jafarizadeh-Malmiri H, Maghsoudi H. *Green Process. Synth.* 2017, 6, 31–42.
[21] Ahdno H, Jafarizadeh-Malmiri H. *Food Bioprod. Process.* 2017, 101, 193–204.
[22] Pereira FB, Guimarães PM, Teixeira JA, Domingues L. *Bioresour. Technol.* 2010, 101, 7856–7863.
[23] Anarjan N, Jaberi N, Yeganeh-Zare S, Banafshehchin E, Rahimirad A, Jafarizadeh-Malmiri H. *J. Am. Oil Chem. Soc.* 2014, 91, 1397–1405.