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Enhancement in the Synthesis of Novel Feruloyl Lipids (Feruloyl Butyryl Glycerides) by Enzymatic Biotransformation Using Response Surface Methodology

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Response surface methodology was successfully employed to optimize lipase-catalyzed synthesis of feruloyl butyryl glycerides (FBGs). The effects of the reaction parameters, including the reaction time, reaction temperature, enzyme concentration, substrate molar ratio, and water activity, and the interaction parameters were examined. The analysis suggested that the conversion of the FBGs was significantly ($p < 0.05$) affected by independent factors of reaction time, reaction temperature, substrate molar ratio, and water activity as well as interactive terms of reaction temperature/reaction time, reaction temperature/enzyme concentration, substrate molar ratio/reaction temperature, water activity/reaction temperature, reaction time/enzyme concentration, and enzyme concentration/water activity. The highest conversion yield of FBGs was 81.2% at the following optimized reaction conditions: reaction temperature of 53.6 °C, reaction time of 5.5 days, enzyme concentration of 50.8 mg/mL, water activity of 0.14, and substrate molar ratio of 2.9. The conversion is higher as compared to that at the conditions before optimization.

KEYWORDS: Feruloyl butyryl glycerides; lipase; response surface methodology; transesterification; optimization

INTRODUCTION

Ferulic acid (FA) is a monophenolic phenylpropanoid present widely in the plant kingdom. Recently, it is of growing particular interest because of its potential biological properties, such as antioxidant, anti-inflammatory, antiviral, and UV filter properties (*1*−*3*). However, due to its hydrophilic nature, applications for this natural compound in oil-based food processing, pharmaceuticals, and cosmetics are limited (*4, 5*). To overcome this limit, modification of FA via its esterification with aliphatic alcohol has been widely investigated (*6*−*9*). Nevertheless, to our knowledge, there are few reports on the modification of FA with triglyceride (*5, 10*).

Ferulyl butyryl glycerides (FBGs), obtained by lipase-catalyzed transesterification of ethyl ferulate (EF) with tributyrin (TB), is a novel feruloylated lipid. It is composed of 1(3)-feruloyl monobutyryl glyceride (FMG) and 1(3)-feruloyl dibutyryl glyceride (FDG) and can exhibit higher solubility and nutritional quality than free ferulic acid in a hydrophobic medium (*11*). Moreover, it is a potential bifunctional compound, because the ferulic acid moiety works as a natural antioxidant, while the butyric acid moiety is a traditional antitumor agent (*2, 12*) (**Figures 1** and **2**).

Chemical synthesis of this kind of FA esters is usually performed with basic or acidic catalysts under reflux. However, with the steadily growing "natural" demand, the synthesis of such esters by lipase-catalyzed reactions under mild conditions has received much attention. Particularly, the development of an optimum enzymatic synthesis procedure to improve the yield of conversion would be more attractive for the manufacturers (*13*).

The earlier work in our laboratory showed that the results were of general interest for the lipase-catalyzed transesterifi-

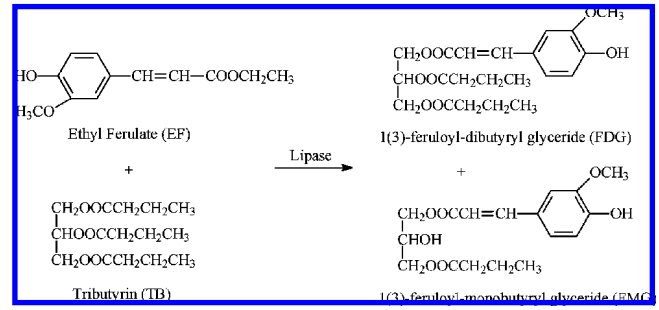

Figure 1. Reaction route of lipase-catalyzed transesterification of EF and TB.

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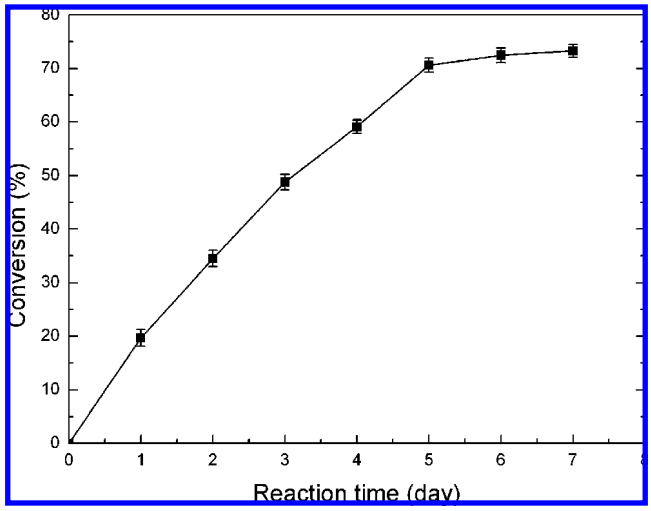

Figure 2. Time course of the lipase-catalyzed synthesis of FBGs. Conditions: temperature, 50 °C; water activity, 0.15; molar ratio of TB to EF, 3; enzyme loading, 40 mg/mL. Vertical bars represent the standard deviation for each data point. Each sample is significantly different ($n = 3$, $p < 0.05$).

cation of EF with TB for the preparation of FBGs (*11*). The present investigation was aimed to enhance our knowledge about the reaction parameters affecting lipase-catalyzed synthesis of FBGs and to optimize the process for enzymatic synthesis.

Response surface methodology (RSM) is an efficient statistical tool for optimization of multiple variables and to predict the best performance conditions for the target value (*14, 15*). It is also a statistical technique useful for designing experiments, building models, and analyzing the effects of independent variables (*14, 16*). Thus, RSM was employed to optimize the synthesis of FBGs via lipase-catalyzed transesterification in this research.

MATERIALS AND METHODS

Materials. Novozyme 435 (*Candida antartica* lipase immobilized on polyacrylic resin, with an activity of 10 000 propyl laurate units (PLU)/g of solid enzyme) was purchased from Novozymes A/S (Bagsvaerd, Denmark). FA (purity >99%) and EF (purity >99%) were purchased from Suzhou Chang Tong Chemical Co., Ltd. (Suzhou, China). TB (purity >99%) was purchased from Tao He Chemical Co., Ltd. (Shanghai, China).

All solvents were of analytical grade and were dried by activated 4 Å molecular sieves before use. All other reagents used were of high purity and were commercially available unless otherwise noted.

Enzymatic Reactions. All enzymatic reactions were carried out in a temperature-controlled incubator shaker at 210 rpm. The procedure for the enzymatic transesterification of FBGs in toluene was described as an example (*1*). The reactions were performed by adding Novozyme 435 (30 mg/mL), EF (0.5 mmol), and TB (1 mmol) in toluene (3 mL) and shaking the reaction mixtures at 45 °C. Samples of the biotransformation were withdrawn at different times and analyzed by high-performance liquid chromatography (HPLC).

HPLC Analysis. Analytical HPLC was performed using a Waters 510 with an Inertsil Ph-3 column (4.6 mm i.d. × 250 mm, 5 μm, GL Sciences, Japan), with a dual-absorbance detector (Waters 2487) at 325 nm. The mobile phase was solution A (water containing 0.1% acetic acid) and solution B (100% methanol), in all cases at 1 mL/min flow of A/B (30:70, v/v). Analysis was carried out at room temperature. The solvents were filtered using Whatman 0.45 μm nylon membrane filters (Sigma-Aldrich) and degassed using a Thermo Separation Products SCM 1000 membrane degasser.

A 10 μL sample was removed from the reaction mixture at set time intervals during the reaction and further diluted 100-fold with methanol. The sample injection volume was 10 μL.

Table 1. Coded Levels for Independent Factors Used in the Experimental Design

factor	symbol	level −2	level −1	level 0	level 1	level 2
reaction temp (°C)	x_1	40	45	50	55	60
reaction time (day)	x_2	3	4	5	6	7
enzyme concn (mg/mL)	x_3	20	30	40	50	60
TB:EF molar ratio	x_4	1	2	3	4	5
a_w	x_5	0.05	0.10	0.15	0.20	0.25

According to previous reports (*4, 5*), the total conversion of FBGs was determined most accurately by measuring the residual EF and FA peak areas by HPLC. The sum of all ferulate species peak areas remained constant over the time course of the reaction, allowing accurate FDG and FMG yields to be calculated as the percentage ratio of the FDG and FMG peak areas to the residual EF and FA peak areas.

Water Activity Pre-Equilibration. Water activity (a_w) is an important consideration for biocatalysis in a nonaqueous medium. It is a better parameter than the water content to determine the amount of water associated with the enzyme and thereby to truly correlate with the enzyme activity (*17*). Among the available methods for controlling a_w, the simple and convenient way is pre-equilibration of the reaction components in the presence of saturated salt solutions, and the method is particularly suitable for the reaction (*18*).

The water activity values of toluene, enzyme, and substrate were adjusted before the reaction was started by the following method: toluene, EF, TB, and Novozyme 435 were separately incubated in a chamber containing a desirable saturated salt solution (*18*), and the system was allowed to reach equilibrium for at least 7 days at 25 °C for the desired a_w. Molecular sieves were used to generate the nearly anhydrous condition ($a_w = 0.05$). The following salts were used in this work: LiCl ($a_w = 0.10$), KAc, $MgCl_2$ ($a_w = 0.15$), $CuCl_2$ ($a_w = 0.20$), and NaCl ($a_w = 0.25$). The water activity of the pre-equilibrated system was measured by a Karl Fischer titrator (Mettler-Toledo, DL31).

Experimental Design. RSM was employed to analyze the operating conditions of lipase transesterification between ethyl ferulate and tributyrin to obtain a high conversion of feruloyl butyryl glycerides. The software Design-Experiment 6.0 (Stat-Ease) was used to design and regress the experimental data. The experimental design was carried out by five chosen independent variables (factors) with five levels (**Table 1**). The investigated factors were the following: reaction temperature (°C), reaction time (day), enzyme concentration (mg/mL), substrate molar ratio (TB:EF), and a_w. For each factor, the experimental range and central point were based on the results of preliminary trials (*11*).

Statistical Analysis. The effect of the reaction time was performed in triplicate, and the level of significance was set at $p < 0.05$. Analysis of variance (ANOVA) and significant differences among the means were tested by one-way ANOVA, using SPSS (version 13.0 for Windows, SPSS Inc., Chicago, IL).

The experimental FBG data (**Table 1**) were best-fitted to the second-order polynomial model ($p < 0.05$) by multiple regression after manual evaluation (p for the first-order model and third-order model was 0.93 and 0.24, respectively, which indicated that both these models were insignificant). The model proposed for the response of Y fitted a second-order polynomial equation as follows:

$$Y = \beta_0 + \sum_{i=1}^{5} \beta_i x_i + \sum_{i=1}^{5} \beta_{ii} x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{ij} x_i x_j \qquad (1)$$

where β_0, β_i, β_{ii}, and β_{ij} are regression coefficients (β_0 is a constant term, β_i is a linear effect term, β_{ii} is a squared effect term, and β_{ij} and is an interaction effect term) and Y is the predicted response value.

RESULTS AND DISCUSSION

Effect of the Reaction Time. The selection of reaction time range needs to be extremely precise in fractional factorial design;

Table 2. Experimental Design and Results of the CCD Design

trial	T (°C)	t (days)	enzyme concn (mg/mL)	TB:EF	a_w	response Y (%)	distribution (%) FDG	distribution (%) FMG
1	45 (−1)	4 (−1)	30 (−1)	2.0 (−1)	0.2 (1)	64.51	30.43	33.15
2	55 (1)	4 (−1)	30 (−1)	2.0 (−1)	0.1 (−1)	60.43	32.97	26.59
3	45 (−1)	6 (1)	30 (−1)	2.0 (−1)	0.1 (−1)	62.58	36.54	24.76
4	55 (1)	6 (1)	30 (−1)	2.0 (−1)	0.2 (1)	60.92	30.54	28.68
5	45 (−1)	4 (−1)	50 (1)	2.0 (−1)	0.1 (−1)	70.32	34.53	34.91
6	55 (1)	4 (−1)	50 (1)	2.0 (−1)	0.2 (1)	56.02	28.77	26.11
7	45 (−1)	6 (1)	50 (1)	2.0 (−1)	0.2 (1)	70.13	35.65	32.97
8	55 (1)	6 (1)	50 (1)	2.0 (−1)	0.1 (−1)	55.32	27.65	25.14
9	45 (−1)	4 (−1)	30 (−1)	4.0 (1)	0.1 (−1)	30.12	12.67	11.98
10	55 (1)	4 (−1)	30 (−1)	4.0 (1)	0.2 (1)	69.26	33.90	33.95
11	45 (−1)	6 (1)	30 (−1)	4.0 (1)	0.2 (1)	64.12	21.14	40.23
12	55 (1)	6 (1)	30 (−1)	4.0 (1)	0.1 (−1)	71.59	32.45	37.86
13	45 (−1)	4 (−1)	50 (1)	4.0 (1)	0.2 (1)	38.11	18.32	17.43
14	55 (1)	4 (−1)	50 (1)	4.0 (1)	0.1 (−1)	67.13	33.45	31.56
15	45 (−1)	6 (1)	50 (1)	4.0 (1)	0.1 (−1)	50.33	20.65	27.62
16	55 (1)	6 (1)	50 (1)	4.0 (1)	0.2 (1)	72.64	36.98	34.31
17	40 (−2)	5 (0)	40 (0)	3.0 (0)	0.15 (0)	52.19	32.12	18.65
18	60 (2)	5 (0)	40 (0)	3.0 (0)	0.15 (0)	71.98	34.65	35.91
19	50 (0)	3 (−2)	40 (0)	3.0 (0)	0.15 (0)	62.56	21.89	38.76
20	50 (0)	7 (2)	60 (−2)	3.0 (0)	0.15 (0)	73.34	34.98	36.32
21	50 (0)	5 (0)	60 (2)	3.0 (0)	0.15 (0)	60.57	32.89	25.87
22	50 (0)	5 (0)	40 (0)	3.0 (0)	0.15 (0)	63.22	29.05	31.97
23	50 (0)	5 (0)	40 (0)	1.0 (−2)	0.15 (0)	60.93	27.15	32.09
24	50 (0)	5 (0)	40 (0)	1.0 (2)	0.15 (0)	50.09	19.65	29.91
25	50 (0)	5 (0)	40 (0)	3.0 (0)	0.05 (−2)	60.24	28.98	30.34
26	50 (0)	5 (0)	40 (0)	3.0 (0)	0.25 (2)	63.54	30.91	30.54
27	50 (0)	5 (0)	40 (0)	3.0 (0)	0.15 (0)	67.06	16.94	49.54
28	50 (0)	5 (0)	40 (0)	3.0 (0)	0.15 (0)	64.89	12.54	51.11
29	50 (0)	5 (0)	40 (0)	3.0 (0)	0.15 (0)	67.12	16.21	49.87
30	50 (0)	5 (0)	40 (0)	3.0 (0)	0.15 (0)	68.98	18.43	48.75
31	50 (0)	5 (0)	40 (0)	3.0 (0)	0.15 (0)	69.29	17.11	50.92
32	50 (0)	5 (0)	40 (0)	3.0 (0)	0.15 (0)	70.12	18.54	50.21

otherwise, the optimal condition of synthesis may not be located inside the experimental region through the analyses of statistics and contour plots. **Figure 1** shows time course for the lipase-catalyzed synthesis of FBGs at 50 °C. The percent molar conversion increased to 70.6% ($p < 0.05$) at 5 days, and there was no significant increase after 5 days. Therefore, the range of reaction time was chosen in the range of 4−6 days in the experiment.

Model Fitting. The combination of RSM and five-factor−five-level central composite experimental design (CCD) was employed to investigate the relationship between the variables and the conversion of FBGs. CCD, which is a 2^k factorial design with star points and center points, was used to fit a full second-order polynomial model. According to statistics theory (*19*), a CCD design of five factors consisted of 32 experiments, including 15 factorial points (cubic point) and 11 axial points (star point) as well as 6 replicates at the center point. The pure error was evaluated by six replications (trials 27−32) at the center of the design. The results at each point based on the experimental design are shown in **Table 2**. In addition, the distribution of FDG and FMG under the experimental condition is also given. The coded values of each factor in parentheses correspond to the real values of the factor levels. For each factor, a conventional level was set to zero as a coded level.

Among the various treatments, the greatest conversion (78.3%) was from trial 20 and the smallest conversion (only 30.1%) was from trial 9. Coefficients of a full model were evaluation by regression analysis and tested for their significance. The insignificant coefficients were eliminated stepwise on the basis of the p value after the coefficients were tested. As indicated in **Table 3**, the independent variables (x_1, x_2, x_4, x_5), interactions (x_1x_4, x_1x_2, x_1x_4, x_1x_3, x_1x_5, x_4x_5, x_2x_3, x_2x_4, x_2x_5, x_3x_5), and quadratic terms (x_1^2, x_3^2, x_4^2, x_5^2) were significant ($p < 0.0001$). The independent variable x_3, the quadratic term x_2^2, and the interaction term x_3x_4 did not produce a significant effect on the conversion within the designed intervals ($p > 0.5$) and were removed from the original model. Thus, the final second-order polynomial equation is

Table 3. Regression Coefficients and Significance ($p < 0.05$) after Backward Elimination for Conversion of FBGs

variable[a]	coefficient	p value
intercept	67.93	<0.0001
x_1	4.28	<0.0001
x_2	3.05	<0.0001
x_3	0.074	0.8430
x_4	−2.44	<0.0001
x_5	1.44	0.0003
x_1^2	−1.48	0.0009
x_2^2	−0.014	0.9658
x_3^2	−1.53	0.0007
x_4^2	−3.12	<0.0001
x_5^2	−1.53	0.0007
x_1x_2	−2.28	0.0003
x_1x_3	−1.17	0.0040
x_1x_4	8.30	<0.0001
x_1x_5	−1.20	0.0211
x_2x_3	−1.13	0.0278
x_2x_4	3.52	<0.0001
x_2x_5	1.76	0.0023
x_3x_4	−0.64	0.7790
x_3x_5	−2.52	0.0001
x_4x_5	1.38	0.0103

[a] See **Table 1**.

$$\text{conversion}\ (\%) = 67.93 + 4.28x_1 + 3.05x_2 - 2.44x_4 + 1.44x_5 - 1.48x_1^2 - 1.53x_3^2 - 3.12x_4^2 - 1.53x_5^2 - 2.28x_1x_2 - 1.17x_1x_3 + 8.30x_1x_4 - 1.20x_1x_5 - 1.13x_2x_3 + 3.52x_2x_4 + 1.76x_2x_5 - 2.52x_3x_5 + 1.38x_4x_5 \quad (2)$$

where x_i is the coded value of each factor.

ANOVA and Adequacy Test of the Model. According to the ANOVA, the coefficient of determination (R^2) of the model was 0.9881, which indicates that the model is suitable to represent the real relationships among the selected reaction parameters (**Table 4**). **Table 4** also shows that the probabilities for regression of the model were significant ($p < 0.0001$), meaning that the models were statistically good, and the models had no lack of fit of the 95% level of significance. As a result, this well-fitting model for conversion of FBGs was successfully established.

Table 4. ANOVA for the Quadratic Model for the Conversion of FBGs

source	sum of squares	DF	mean square	*F* value	*p* > *F*
model	2906.71	20	145.34	45.78	<0.0001
residual	34.99	11	3.17		
lack of fit	16.52	6	2.75	0.75	0.6372
pure error	18.40	5	3.68		
total	2941.36	31			
R^2 [a]	0.9881				

[a] $R^2 = 0.9881$.

Mutual Effect of the Parameters. A clear interpretation of the effects of variable interaction on the conversion of FBGs can be seen in the response contour plots (**Figure 3**).

Figure 3a depicts the effects of water activity and substrate molar ratio and their mutual interaction on the conversion of reaction at 5 days, 50 °C, and 40 mg/mL enzyme concentration. Many research groups have reported that water activity presented in the reaction system was one of the most significant factors (*20*). On one hand, a certain amount of water is essential to keep the lipase activity, and almost all kinds of lipase have their own optimum a_w (*21*); on the other hand, an excess of water in the reaction media will inhibit the synthetic reaction while promoting the hydrolysis of the acylated products (*22*) and the acyl donors (*23*). As presented in **Figure 3a**, both the higher water activity ($a_w > 0.18$) and lower water activity ($a_w < 0.13$) had negative effects on the conversion of reaction. Also, the

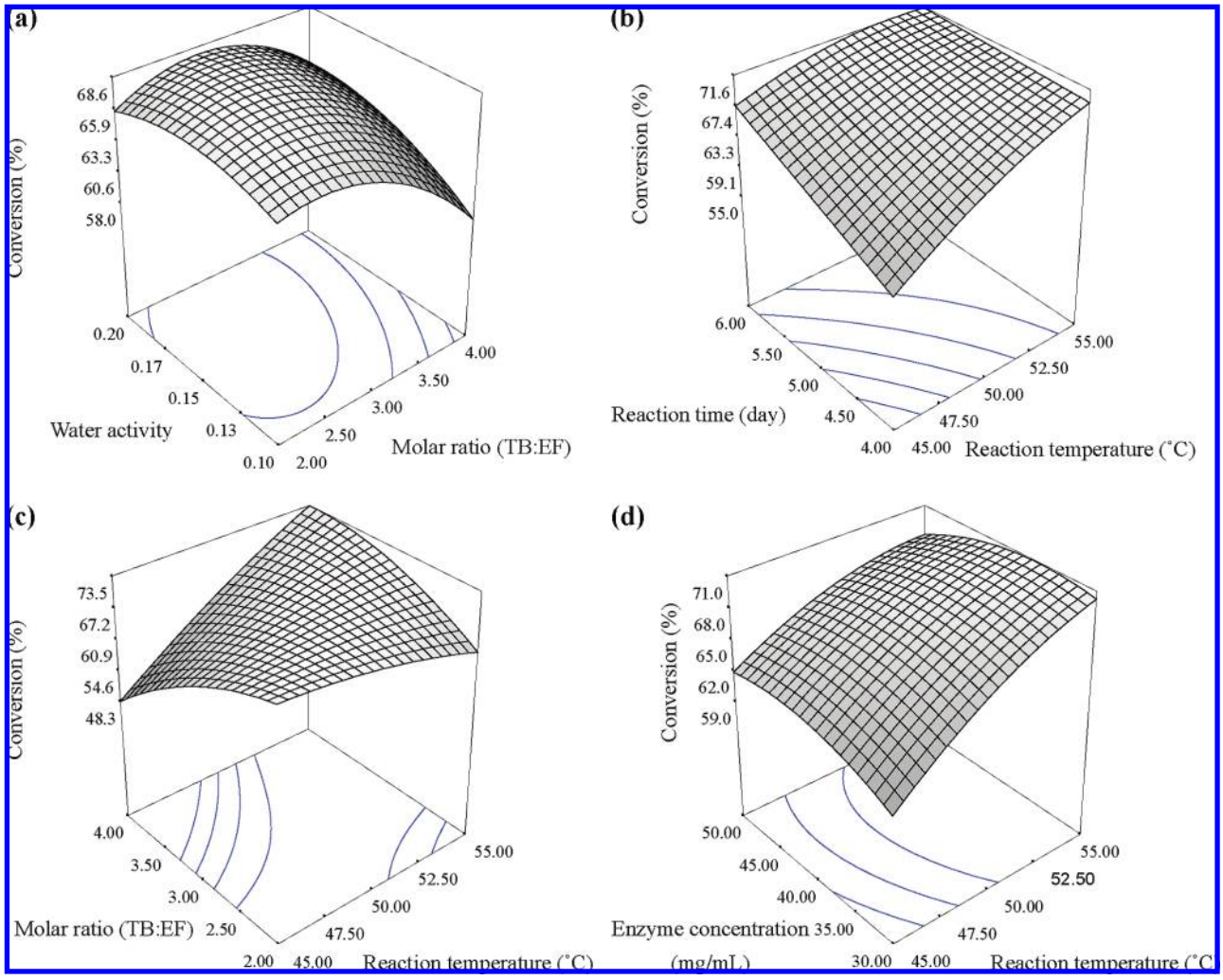

Figure 3. Response surface plots: (**a**) effect of the water activity and substrate molar ratio and their mutual interaction on production of FBGs, with other parameters (water activity, substrate molar ratio, and enzyme concentration) being set constant at the 0 level; (**b**) effect of the reaction time and reaction temperature and their mutual interaction on prodution of FBGs, with other parameters (water activity, reaction time, and enzyme concentration) being set constant at the 0 level; (**c**) effect of the substrate molar ratio and reaction temperature and their mutual interaction on production of FBGs, with other parameters (water activity, reaction time, and enzyme concentration) being set constant at the 0 level; (**d**) effect of the enzyme concentration and reaction temperature and their mutual interaction on production of FBGs, with other parameters (water activity, reaction time, and substrate molar ratio) being set constant at the 0 level.

effect of the substrate molar ratio on the conversion was significant ($p < 0.05$). The conversion increased as the substrate molar ratio was increased from 1 to 3; however, when the substrate molar ratio is larger than 3, it could lead to a decrease in conversion. These results indicate that the excess of TB would lead to an increased limitation of the substrate−enzyme reaction sites, which leads to a decrease in conversion. It was concluded that a high conversion could be obtained by combining $a_w = 0.15$ and a substrate molar ratio of TB to EF of 3.

Figure 3b shows the effect of the reaction time and reaction temperature on the conversion of the reaction with a 40 mg/mL enzyme concentration, a substrate molar ratio of TB to EF of 3, and $a_w = 0.15$. As presented in **Figure 3b**, an increasing reaction temperature could raise the conversion of the reaction strongly. It was indicated that a high temperature can activate the substrate molecules, reduce the viscosity of the reaction, and lead to a higher reaction rate. Also, within the given range (4−6 days) of reaction time, the conversion yield of the reaction increased almost linearly with an increase in the reaction time. This phenomenon has also been reported by Dossat et al. (*24*). Not surprisingly, the conversion of the reaction peaked with a maximum conversion of 69.4% at 55 °C and 6 days. From the analysis of the response surface plots, both the reaction temperature and reaction time exhibited a positive influence on the response surface.

Figure 3c denotes the effect of the substrate molar ratio and reaction temperature on the conversion of the reaction at 5 days, a 40 mg/mL enzyme concentration, and $a_w = 0.15$. The interaction term of the substrate molar ratio and reaction temperature played an important role in the process of the reaction as was evident from its first-order effect ($p < 0.05$). When the reaction temperature and substrate molar ratio increased to 55 °C and 4, respectively, the conversion of the reaction achieved was 72.7%. It must be pointed out that, at low temperature (<50 °C), conversion declined with increasing substrate molar ratio, which could be attributed to the fact that there was great resistance of the viscosity of the reaction caused by a high amount of TB when the temperature was low.

Figure 3d shows the enzyme concentration and temperature effect on the conversion of the reaction at 5 days, a substrate molar ratio of TB to EF of 3, and $a_w = 0.15$. It was observed that the interaction between the reaction temperature and enzyme concentration obviously affects the conversion, and this was also confirmed by the p value ($p = 0.004$). At any given enzyme concentration in the investigated range (30−50 mg/mL), an increase in the temperature from 45 to 55 °C could lead to an increase in conversion. The reaction with a high reaction temperature (55 °C) and enzyme amount (50 mg/mL) favored maximal conversion, which indicated the same report from Novo Nordisk Bioindustrials that the enzyme is stable in an organic solvent at higher temperature (40−60 °C).

Model Verification. Within the experimental range studied, optimum conditions for synthesis of FBGs were predicted using the optimization function of Design Expert. Two sets of predicted reaction conditions are presented in **Table 5** along with their predicted and actual values. To validate the predicted results, experiments using the improved formula were performed, and the observed values are shown in **Table 5**. On the basis of the solution given by the design, two runs of experiments were established at the fixed conditions. The experimental values were found to be in reasonable agreement with the predicted ones, which confirmed the validity and adequacy of the predicted models. Moreover, the conversion is higher as compared to the conditions before optimization (trials 27−30). Thus, it could be concluded that the optimization of lipase-catalyzed synthesis of FBGs by Novozyme 435 was successfully developed by RSM. The experimental conditions allowed a quantitative and maximum conversion of FBGs.

Table 5. Optimum Conditions Founded by the Model for the Conversion of FBGs

	run 1	run 2
reaction temp (°C)	53.62	53.1
reaction time (day)	5.5	6
enzyme concn (mg/mL)	50.76	36.25
TB:EF molar ratio	2.91	3.12
a_w	0.14	0.20
predicted value (%)	79.92	64.69
experimental value (%) ± SD[a]	81.21 ± 0.7	62.04 ± 0.9

[a] Standard deviation of triplicate determinations from different experiments.

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