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ScienceDirect
Food Chemistry 102 (2007) 336–342
Food Chemistry
www.elsevier.com/locate/foodchem

Modeling and optimization of lipase-catalyzed synthesis of phytosteryl esters of oleic acid by response surface methodology

Byung Hee Kim, Casimir C. Akoh *

Department of Food Science and Technology, The University of Georgia, Food Science Building, Room 211, Athens, GA 30602-7610, USA

Received 2 March 2006; received in revised form 26 April 2006; accepted 17 May 2006

Abstract

Enzymatic esterification of phytosterols with oleic acid to produce phytosteryl esters was performed in hexane. Response surface methodology was used to model the reaction. *Candida rugosa* lipase was the biocatalyst for the reaction. The reaction factors investigated were temperature (Te = 35–55 °C), reaction time (t = 4–24 h), substrate molar ratio (Sr = 1–3, oleic acid:phytosterols), and enzyme amount (En = 2–10%). Well-fitting quadratic polynomial regression model for degree of esterification (DE) was established after regression analysis with backward elimination and verified by a χ^2 test. All factors investigated positively affected DE, with t having the greatest effect followed by En, Sr, and Te. The quadratic terms of t, Sr, and En showed negative effects on DE, whereas, that of Te had no effect on DE. Optimal reaction conditions were: Te, 51.3 °C; t, 17.0 h; Sr, 2.1; En, 7.2% and DE was 97.0 mol% under these conditions.

Keywords: *Candida rugosa* lipase; Esterification; Phytosterols; Phytosteryl esters of fatty acids; Response surface methodology

1. Introduction

Phytosterols are sterols derived from plant sources, such as vegetable oils and cereals and have similar structure with animal tissue sterol, cholesterol. However, phytosterols are known to have a hypocholesterolemic effect by lowering plasma total and low density lipoprotein (LDL) cholesterol levels without affecting plasma high density lipoprotein (HDL) cholesterol concentration (Beveridge, Haust, & Connel, 1964; Lees, Mok, Lees, McCluskey, & Grundy, 1977; Pollak, 1953; Wester, 2000). It is generally believed that plasma total and LDL cholesterol levels can be reduced up to 10–20% with a dose of 1.5–3.0 g phytosterols/day in humans (Hendriks, Weststrate, van Vliet, & Meijer, 1999; Jones, MacDougall, Ntanios, & Vanstone, 1997; Katan et al., 2003; Ling & Jones, 1995; Miettinen & Gylling, 2004; Miettinen, Puska, Gylling, Vanhanen, & Vartiainen, 1995; Nestel, Cehun, Pomeroy, Abbey, & Weldon, 2001; Noakes et al., 2002; Weststrate & Meijer, 1998).

Because of such health beneficial attributes of phytosterols, their application as a dietary supplement has been recently attempted in various types of food products such as margarine, spread, salad dressing, edible oil, and milk. However, phytosterols have limitations in usage as a dietary supplement since they possess very low solubility in edible oil and have very high melting point. Therefore, to overcome such problems of free forms of phytosterols, phytosteryl esters (i.e., fatty acid (FA) ester forms of phytosterols), are preferred in food formulations. Since phytosteryl esters have much greater solubility in oils and much lower melting point as compared to the corresponding phytosterols, they can be easily incorporated into a wide variety of diets and fat-based food products and provide an easy means of intake of the daily amount of phytosterol needed for sufficient reduction of cholesterol absorption without changing the taste of the final product. Moreover, many studies in recent years have shown that phytosteryl esters can also effectively reduce plasma total

* Corresponding author. Tel.: +1 706 542 1067; fax: +1 706 542 1050.
E-mail address: cakoh@uga.edu (C.C. Akoh).

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doi:10.1016/j.foodchem.2006.05.025

and LDL cholesterol levels in a similar manner as phytosterols (Jones et al., 1997; Law, 2000; Neil, Meijer, & Roe, 2001; Weststrate & Meijer, 1998).

Phytosteryl esters of FAs are presently synthesized by chemical esterification and transesterification. However, the chemical method involves some problems such as the formation of side products (e.g., dehydrated or oxysterols) and staining (Negishi, Hidaka, Takahashi, & Kunita, 2003; Villeneuve et al., 2005). Hence, in recent years, several enzymatic procedures using lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) obtained from diverse kinds of microbial sources for the preparation of phytosteryl esters of FAs have been developed to overcome such shortcomings of chemical method (Negishi et al., 2003; Shimada et al., 1999; Villeneuve et al., 2005; Vu, Shin, Lim, & Lee, 2004; Weber, Weitkamp, & Mukherjee, 2002; Weber, Weitkamp, & Mukherjee, 2001). The lipase-catalyzed esterification and transesterification were carried out successfully in monophasic media (organic solvents) (Villeneuve et al., 2005; Vu et al., 2004) or in multiphasic media (oil/water two-phase) (Shimada et al., 1999), and furthermore in oil itself (Negishi et al., 2003; Weber et al., 2002; Weber et al., 2001), with/without removing water, which is generated during the esterification between FAs and phytosterols, by the use of water-trapping agents (e.g., KOH pellet and molecular sieve) or reduced pressure conditions. However, for future industrial scale enzymatic production of phytosteryl esters of FAs, it would be beneficial to simplify the reaction conditions as much as possible. In our current study, we successfully performed small scale synthesis of phytosteryl esters of FAs in high yields without removal of water generated during the reactions. The synthesis with free phytosterols and FAs was carried out in hexane under mild reaction conditions (low temperature ~55 °C and short reaction time ~24 h). So far there are few studies on elucidating the effect of several reaction parameters on phytosteryl ester synthesis as well as modeling the reactions catalyzed by lipases.

The objective of our study was to model the lipase-catalyzed esterification reaction between phytosterols and oleic acid to produce phytosteryl esters of oleic acid and to optimize the reaction conditions. The effects of four reaction parameters (temperature, reaction time, substrate molar ratio, and enzyme amount) on the degree of esterification were evaluated, and quadratic polynomial model equations for the degree of esterification were also established by response surface methodology (RSM), and then the optimal reaction conditions were proposed.

2. Materials and methods

2.1. Materials

β-Sitosterol (purity > 40%) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). The β-sitosterol was analyzed and found to contain three major phytosterol analogues (β-sitosterol 42.7 mol%, campesterol 27.1 mol%, dihydrobrassicasterol 25.2 mol%; total, 95.0 mol%) by GC in our laboratory. Oleic acid (C18:1, purity > 99%) was also purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Powdered *Candida rugosa* lipase (CRL) type VII (EC 3.1.1.3) was the product (Cat. No.: L 1754) of Sigma–Aldrich Co. (St. Louis, MO, USA). *n*-Hexane and anhydrous diethyl ether were purchased from J.T. Baker (Philipsburg, NJ, USA). Plant sterol mixture and Corowise™ phytosteryl esters were products of Matreya Inc. (Pleasant Gap, PA, USA) and Cargill Inc. (Minneapolis, MN, USA), respectively. All other reagents used were of analytical grades and purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Experimental design for RSM

Factors considered important were temperature (Te = 35–55 °C), reaction time (t = 4–24 h), substrate molar ratio; i.e., oleic acid to total phytosterols molar ratio (Sr = 1–3), and enzyme amount; i.e., weight percent of total substrates (En = 2–10%). RSM was used to optimize reaction parameters. Central composite design (CCD) was adopted in this study. CCD is a 2^k factorial design with star points and center points. Twenty seven experimental settings consisting of 8 star points (star distance is 0) and 3 center points were generated with 4 factors and 3 levels by the principle of RSM using commercial software, *Modde* 5.0 (Umetrics, Umeå, Sweden). The quadratic polynomial regression model was assumed for predicting Y variable (DE = degree of esterification of phytosterols with oleic acid). The model proposed for the response of Y fitted Eq. (1) as follows:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \quad (1)$$

where Y is response variable (DE, mol%). β_0, β_i, β_{ii}, and β_{ij} are constant coefficients of intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables (Te, t, Sr, and En).

2.3. Lipase-catalyzed esterification

Fifty milligrams of phytosterols was mixed with different milligrams of oleic acid corresponding to the different substrate molar ratios generated by RSM, in screw-capped test tubes. Different amounts of lipase, which were generated by RSM, and then 1.5 ml of *n*-hexane were subsequently added. The reaction was performed in an orbital shaking water bath at 200 rpm at different temperatures and for different time periods generated by RSM, as indicated in Table 1. The reaction was stopped by cooling in running cold water, and then 3 ml of anhydrous diethyl ether was added. The mixtures were vortexed for 30 s, centrifuged at 2000 rpm for 3 min, and then the solvent containing reactants were passed through sodium sulfate column to remove the lipase and water generated during reaction.

Table 1
Central composite design arrangement and response for the *Candida rugosa* lipase (CRL)-catalyzed esterification to synthesize phytosteryl esters of oleic acid[a]

Exp. no.	Factors				Response
	Te (°C)	*t* (h)	Sr	En (%)	DE (mol%)
1	35	4	1	2	47.8 ± 0.3[b]
2	55	4	1	2	60.4 ± 4.1
3	35	24	1	2	76.2 ± 3.8
4	55	24	1	2	92.2 ± 0.4
5	35	4	3	2	69.4 ± 3.2
6	55	4	3	2	77.5 ± 3.2
7	35	24	3	2	89.7 ± 3.8
8	55	24	3	2	96.6 ± 0.3
9	35	4	1	10	76.3 ± 3.4
10	55	4	1	10	87.0 ± 0.4
11	35	24	1	10	95.5 ± 0.0
12	55	24	1	10	94.5 ± 0.8
13	35	4	3	10	91.6 ± 1.8
14	55	4	3	10	93.3 ± 1.1
15	35	24	3	10	96.4 ± 1.2
16	55	24	3	10	96.4 ± 0.5
17	35	14	2	6	96.5 ± 0.8
18	55	14	2	6	97.1 ± 0.2
19	45	4	2	6	86.6 ± 1.8
20	45	24	2	6	96.5 ± 0.3
21	45	14	1	6	91.1 ± 0.7
22	45	14	3	6	96.7 ± 0.6
23	45	14	2	2	89.0 ± 1.0
24	45	14	2	10	97.1 ± 0.3
25	45	14	2	6	95.8 ± 0.3
26	45	14	2	6	96.6 ± 0.3
27	45	14	2	6	97.2 ± 0.9

[a] Te = temperature; *t* = reaction time; Sr = substrate molar ratio (oleic acid to phytosterols); En = enzyme amount; DE = degree of esterification of phytosterol with oleic acid to form phytosteryl esters of oleic acid.
[b] Mean ± SD, $n = 2$.

Diethyl ether (4 ml) was added two more times to recover the reaction products thoroughly. After the extraction, the solvents were completely evaporated under the flow of nitrogen. The sample was redissolved in chloroform (4 ml) and then 1 μl of the solution was used for gas chromatographic (GC) analysis.

2.4. GC analysis

The composition of the reaction products was analyzed by GC. A Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard Co., Avondale, PA, USA), equipped with a FID and a fused silica capillary column (DB-5ht, 30 m × 0.25 mm i.d., Agilent Technologies., Deerfield, IL, USA) was used. The carrier gas was helium and the total gas flow rate was 23 ml/min. The injector and detector temperatures were maintained at 350 °C, respectively. The column was heated initially at 180 °C and programmed to increase to 300 °C at the rate of 3 °C/min. The column temperature was then programmed to increase to 380 °C at the rate of 10 °C/min and held at 380 °C for 5 min. The phytosterols and phytosteryl esters of oleic acid were identified using plant sterol mixture and Corowise ™ phytosteryl esters (major phytosterols: sitosterol *ca*. 58 mol%, campesterol *ca*. 28 mol%; major FAs: oleic acid *ca*. 56 mol%, linoleic acid *ca*. 20 mol%) and their relative contents were calculated as mol%. With the GC operation conditions above, the retention time of each compound was as follows: oleic acid (9.53 min); campesteryl ester of oleic acid (27.41 min); dihydrobrassicasteryl ester of oleic acid (28.13 min); β-sitosteryl ester of oleic acid (29.39 min); campesterol (32.26 min); dihydrobrassicasterol (32.97 min) and β-sitosterol (34.22 min).

2.5. Calculation of degree of esterification

The degree of esterification (mol%) of phytosterols with oleic acid to form phytosteryl esters of oleic acid was calculated from the GC profile of reactants using the following Eq. (2):

$$\text{Degree of esterification (DE, mol\%)} = \frac{B}{1.638 \times A + B} \times 100 \quad (2)$$

where A = peak area of total phytosterols (i.e., campesterol + dihydrobrassicasterol + β-sitosterol); B = peak area of total phytosteryl esters of oleic acid (i.e., campesteryl ester of oleic acid + dihydrobrassicasteryl ester of oleic acid + β-sitosteryl ester of oleic acid); 1.638 = ratio of average molecular weight of total phytosteryl esters of oleic acid to average molecular weight of total phytosterols.

2.6. Statistical analysis

All data were analyzed with the assistance of commercial software, Modde 5.0 (Umetrics, Umeå, Sweden) and were presented as the mean ± SD. The significant second-order coefficients were selected by regression analysis with backward elimination. Then, the fit of the model was evaluated by coefficients of determination (R^2 and Q^2 values) and a test for lack of fit, which was performed by comparing mean square (MS) lack of fit to MS pure experimental error, from the analysis of variance (ANOVA). The model equation established was finally proposed after verification by a chi-square test.

3. Results and discussion

The ultimate goal of our study was to model the degree of esterification (DE) of total phytosterols with oleic acid when CRL was used as the biocatalyst for the esterification reaction. In our study, we used phytosterol mixture containing three kinds of major phytosterols (β-sitosterol, campesterol, dihydrobrassicasterol) as the substrate. Weber et al. (2001) reported that the activity of CRL can be affected by types of phytosterols in the synthesis of phytosteryl esters of FAs. This indicates that the CRL-catalyzed esterification to synthesize FA esters of phytosterols is a substrate dependent enzyme reaction. In our study, β-sitosterol and campesterol showed similar DE with oleic acid at all reaction conditions investigated; whereas

dihydrobrassicasterol showed relatively lower DE. For example, in the experiment No. 1 (from Table 1), DE of each phytosterol analogue was as follows: β-sitosterol 49.7 mol%, campesterol 52.1 mol%, and dihydrobrassicasterol 38.6 mol%. However, in the experimental sets showing very high DE of total phytosterols, all the 3 kinds of phytosterols showed almost the same level of DE (e.g., experiment No. 27 from Table 1: β-sitosterol 97.4 mol%, campesterol 94.8 mol%, and dihydrobrassicasterol 97.0 mol%). Thus, we succeeded in modeling DE of individual phytosterol analogues as well as DE of total phytosterols. We note that the DE of total phytosterols was the main focus of this paper.

3.1. Model fitting

RSM was applied to model the DE of phytosterols with oleic acid to produce phytosteryl esters of oleic acid, with 4 reaction parameters: temperature (Te), reaction time (t), substrate molar ratio (Sr), and enzyme amount (En). RSM enabled us to obtain sufficient information for statistically acceptable results using reduced number of experimental sets, and is an efficient method to evaluate the effects of multiple parameters, alone or in combination, on response variables (Huang & Akoh, 1996; Shieh, Akoh, & Koehler, 1995; Xu et al., 1998). Table 1 lists the levels of DE at each of the 27 experimental sets generated by the principles of RSM used in this study and the levels ranged from as low as 47.2 to as high as 97.2 mol%. The best-fitting models were determined by multiple linear regression (MLR) and backward elimination. The fits of the models were evaluated by coefficients of determination (R^2 and Q^2 values) and a test for lack of fit from ANOVA (Table 2). According to the ANOVA, R^2, which means the fraction of the variation of the response explained by the model, and Q^2, which indicates the fraction of the variation of the response predicted by the model, were 0.973 and 0.884, respectively (Table 2). Table 2 also showed that the probabilities for the regression of the model were significant ($P < 0.001$), meaning that the models were statistically good, and the models had no lack of fit at 95% level of significance (Table 2). The normal probability plot (data not shown) and the observed values vs. predicted values plot (Fig. 1) also showed almost linear distribution, which is indicative of a good model. As a result, well-fitting models for DE were successfully established.

Table 2
ANOVA table[a]

	df	SS	MS	F-values	P-values
Total	27	214,072	7928.6		
Constant	1	209,969	209,969		
Total corrected	26	4103.2	157.82		
Regression	12	3991.2	332.60	41.564	<0.001
Residual	14	112.03	8.002		
Lack of fit (model error)	12	111.04	9.2534	18.757	0.052
Pure error (Replicate error)	2	0.9867	0.4933		
R^2	0.973				
Q^2	0.884				

[a] Abbreviations: df, degrees of freedom; SS, sum of squares; MS, mean square.

3.2. Effects of parameters

Table 3 lists the significant ($P < 0.05$) regression coefficients of the established model equation. Although among them, two terms (Sr * Sr and Te * Sr) were significant at 90% level, they were not removed by backward elimination in the process of model fitting described before to enhance the fitness of model. All 4 reaction parameters investigated positively affected DE, with t having the greatest effect followed by En, Sr, and Te. The quadratic terms of t, Sr, and En showed negative effects on DE; whereas, the quadratic term of Te had no significant ($P < 0.05$) effect on DE. All

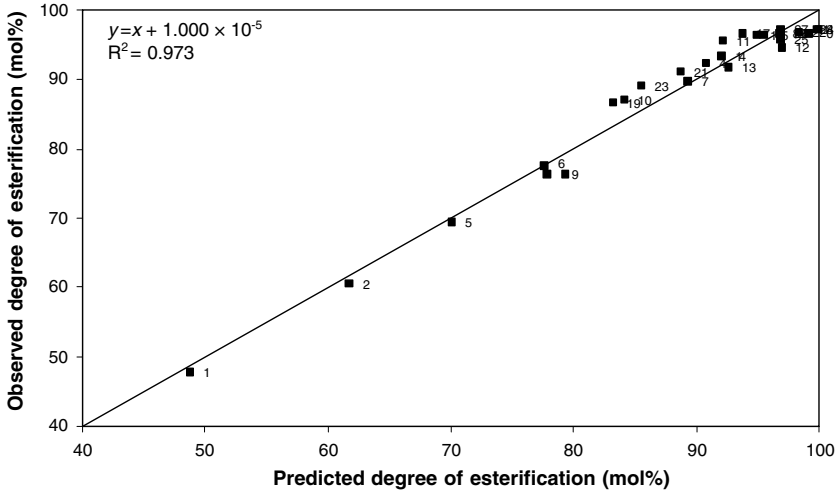

Fig. 1. Plot showing relationships between observed values and values predicted by the model. Numbers inside the graph represent experimental numbers. The almost linear distribution of the experimental numbers is indicative of a good model.

Table 3
Significant ($P < 0.05$) regression coefficients of the second-order polynomials after backward elimination for the *Candida rugosa* lipase (CRL)-catalyzed esterification to synthesize phytosteryl esters of oleic acid[a]

Variables	Coefficients	*P*-values
Intercept	96.880	5.069×10^{-21}
Te	3.089	3.876×10^{-4}
t	8.006	9.285×10^{-9}
Sr	4.811	4.454×10^{-6}
En	7.183	3.683×10^{-8}
$t * t$	−5.631	4.949×10^{-3}
Sr * Sr[b]	−3.281	7.269×10^{-2}
En * En	−4.131	2.839×10^{-2}
Te * Sr[b]	−1.350	7.698×10^{-2}
Te * En	−2.013	1.296×10^{-2}
t * Sr	−2.475	3.537×10^{-3}
t * En	−4.063	5.073×10^{-5}
Sr * En	−2.013	1.296×10^{-2}

[a] See Table 1 for description of abbreviations.
[b] Regression coefficient which is significant at $P < 0.1$.

the significant ($P < 0.05$) interaction terms negatively affected DE and the order of effect is as follows: t * En, t * Sr, Sr * En = Te * En, and Te * Sr.

Fig. 2(a) shows that within the given range (35–55 °C) of Te, DE increased almost linearly with the increase in Te. Tenkanen, Kontkanen, Isoniemi, Spetz, and Holmbom (2002) reported that CRL had optimal activity below 55 °C at pH 5–7 when applied to the hydrolysis of phytosteryl esters. Therefore, our esterification result was in close agreement with their hydrolysis result. However, for better clarification of optimal temperature of CRL in the esterification reaction between phytosterols and oleic acid, further investigation on the synthesis of phytosteryl esters of oleic acid above 55 °C would be required. Fig. 2(b)–(d) illustrate that the rate of increase in DE was decreased, respectively, after center points of each parameter (t, 14 h; Sr, 2; En, 6%) even though overall DE continued to increase. It is possible that equilibrium was reached at these center points.

3.3. Model verification

A chi-square test using eight additional experimental sets chosen from the given ranges of reaction parameters was performed to examine the adequacies of the model

Table 4
Model verification by chi-square (χ^2) test[a]

Exp. no.	Factors				Responses	
	Te (°C)	t (h)	Sr	En (%)	DE (mol%)	
					Observed	Predicted
1	35.9	4.7	1.2	2.1	50.6 ± 4.9[b]	55.0
2	40.0	7.5	1.1	2.6	71.9 ± 1.0	65.0
3	50.6	5.7	1.4	3.5	80.5 ± 4.3	75.0
4	50.1	4.0	1.0	8.0	77.4 ± 0.6	80.0
5	37.1	18.9	1.2	3.7	84.8 ± 1.8	85.0
6	38.6	21.4	1.1	5.2	93.6 ± 2.0	90.0
7	48.6	8.9	1.7	9.0	92.0 ± 3.6	95.0
8	55.0	9.8	2.5	8.2	96.6 ± 0.0	99.0
					$\chi^2 = 1.87$[c]	

[a] See Table 1 for description of abbreviations.
[b] Mean ± SD, $n = 2$.
[c] $\chi^2 = \sum$ [(Observed value − Predicted value)^2/Predicted value]; cutoff point was 14.07 at $\alpha = 0.05$, df = 7.

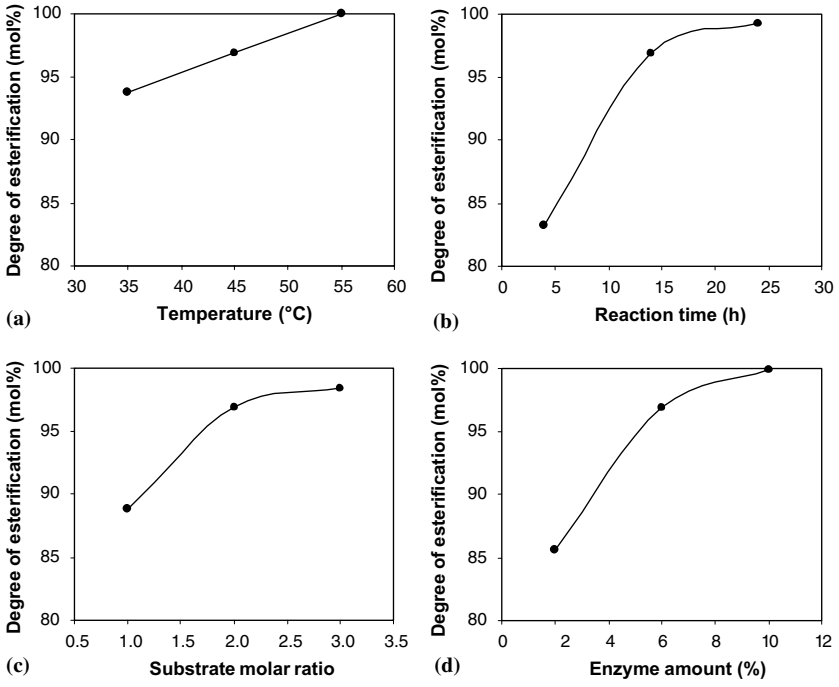

Fig. 2. Prediction plots for degree of esterification of phytosterols with oleic acid to produce phytosteryl esters of oleic acid by the effects of main parameters: (a) temperature (Te); (b) reaction time (t); (c) substrate molar ratio (Sr); (d) enzyme amount (En). Factors setup: t 14 h, Sr 2, En 6% for (a); Te 45 °C, Sr 2, En 6% for (b); Te 45 °C, t 14 h, En 6% for (c); Te 45 °C, t 14 h, Sr 2 for (d).

established. The chi-square test for DE indicated that there were no significant ($P < 0.05$) difference between the observed and predicted values since the chi-square value (1.87) was much smaller than 14.07, cutoff point at $\alpha = 0.05$ and df = 7 (Table 4).

3.4. Optimization of reaction conditions

Contour plot is generally used to evaluate the relationships between parameters and to predict the result under given conditions. However, it is complicated to analyze the interaction between parameters in this study due to the existence of many interaction terms as shown in Table 3.

Instead, we used contour plots for optimizing the conditions of esterification reaction. Six contour plots between four parameters were constructed and minimum level of each parameter which would enable us to reach the maximum level (theoretically 100%) of DE was predicted and pointed by using dotted arrows on each contour plot (Fig. 3). From the result, it can be concluded that optimal conditions for synthesis of phytosteryl esters of oleic acid were: Te, 51.3 °C; *t*, 17.0 h; Sr, 2.1; En, 7.2%. When one additional esterification reaction was conducted under the

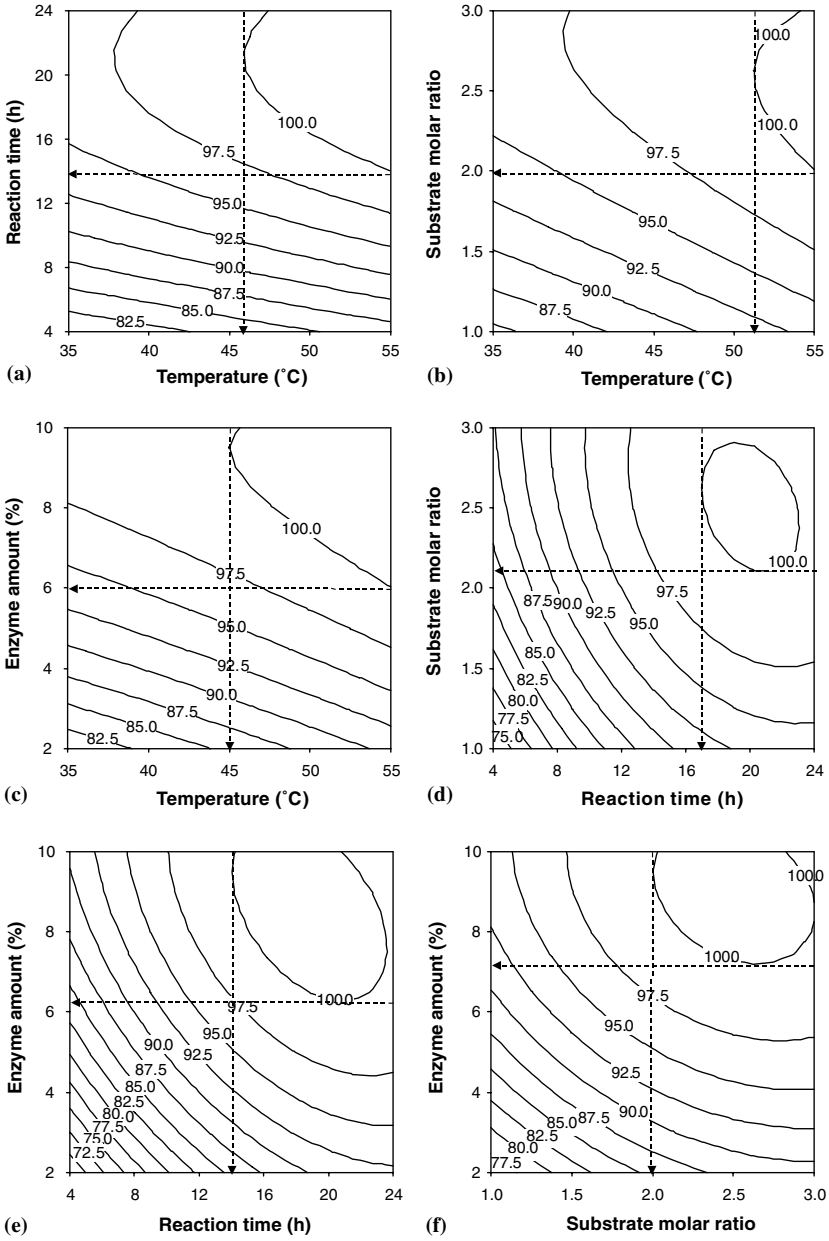

Fig. 3. Contour plots between two parameters for degree of esterifcation of phytosterols with oleic acid to produce phytosteryl esters of oleic acid: (a) temperature (Te) and reaction time (*t*); (b) Te and substrate molar ratio (Sr); (c) Te and enzyme amount (En); (d) *t* and Sr; (e) *t* and En; (f) Sr and En. Factors setup: Sr 2, En 6% for (a); *t* 14 h, En 6% for (b); *t* 14 h, Sr 2 for (c); Te 45 °C, En 6% for (d); Te 45 °C, Sr 2 for (e); Te 45 °C, *t* 14 h for (f).

established optimal conditions, as expected, DE (97.0 ± 0.5 mol%) near 100 mol% was achieved.

4. Conclusion

The modeling of CRL-catalyzed esterification reaction to synthesize phytosteryl esters of oleic acid as a possible cholesterol lowering agent was successfully performed. By using the established model, the degrees of effect of four main reaction parameters (temperature, reaction time, substrate molar ratio, and enzyme amount) were elucidated at given ranges and optimized reaction conditions were obtained. In addition, very high DE (up to 97.2 mol%) of phytosterol with oleic acid was achieved even though our reaction conditions are shown to be simple and mild (i.e., monophasic media of hexane, low temperature ~55 °C, short reaction time ~24 h, and without the use of water-trapping agents or reduced pressure system) compared to other previous works (Negishi et al., 2003; Shimada et al., 1999; Weber et al., 2002; Weber et al., 2001; Vu et al., 2004). The phytosteryl esters of oleic acid produced in this study are expected to have lower melting point and greater solubility in oils compared to the corresponding phytosterols with free hydroxyl groups.

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