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ORIGINAL PAPER

Serial connection of packed-bed reactors with different reaction temperatures: enhanced operational stability for enzymatically interesterified *trans*-free lipid production

Jong Won Won · Kyung Min Park · Seung Jun Choi · Pahn-Shick Chang

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Abstract A packed-bed enzyme reactor (PBER) system using immobilized lipase was successfully developed for the continuous production of *trans*-free interesterified oil with fully hydrogenated canola oil (FHCO) and soybean oil. Optimal interesterification conditions were established by determining the effects of reaction temperature, flow rate, and substrate weight ratio on conversion degree. It was found out that flow rate had a significant effect on conversion degree. Optimal conditions were as follows: FHCO concentration, 38.2 %; flow rate, 0.4 mL/min; and reaction temperature, 81.4 °C. Predicted and experimental conversion degrees were 89.5 and 87.1 %, respectively. The operational stability of the immobilized lipase and conversion degree using the serially connected PBER was higher than a single PBER. The adjustment of the interesterification conditions made possible to produce the interesterified oils having various melting range. This study demonstrates that a multi-stage PBER system can be used to enzymatically produce *trans*-free interesterified oils with various polymorphic properties.

J. W. Won · K. M. Park · P.-S. Chang (✉)
Department of Agricultural Biotechnology,
Seoul National University, Seoul 151-742, Republic of Korea
e-mail: pschang@snu.ac.kr

S. J. Choi
Department of Food Science and Technology,
Seoul National University of Science and Technology,
Seoul 139-743, Republic of Korea

P.-S. Chang
Research Institute for Agriculture and Life Sciences,
Center for Food Safety and Toxicology,
Center for Agricultural Biomaterials, Seoul National University,
Seoul, Republic of Korea

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Introduction

Recently, consumer demand has increased for value-added and healthier fats and/or oils, which is challenging for the edible oil industry. However, natural fats and oils often fall short of consumer preferences due to their fatty acid composition and regiochemical or stereochemical structure [1]. The limited application of natural oils has forced the edible oil industry to research areas such as lipid modification. To produce fats and oils with suitable physical and healthy characteristics, various lipid modification methods have been developed and applied [2].

Given the growing demand for value-added and healthy food products, many researchers have studied fat and oil modification techniques, including hydrogenation, transesterification, and interesterification [3]. However, during partial or full hydrogenation, some *cis*-double bonds are isomerized into the *trans*-form. These *trans* fatty acids are associated with coronary heart disease and diabetes [4]. Because of these adverse health effects, the demand for healthy, *trans* fat-free products is rapidly increasing. Interesterification is an alternative technology that can replace the conventional hydrogenation process and reduce or eliminate *trans* fatty acids. Interesterification exchanges fatty acids on the glycerol backbone. The rearrangement of certain fatty acids within triacylglycerol molecules changes their physical characteristics for food applications. The chemical properties of the original fat are relatively unaffected, and the fatty acids' inherent properties are unchanged. Also, there is no *cis–trans* isomerization.

Interesterification can be performed either chemically or enzymatically [5, 6]. Chemical interesterification generally leads to full randomization. Since the reaction occurs rapidly, it is difficult to terminate at partial interesterification. In enzymatic interesterification, the degree of conversion to suitable oil products with the desired physical and chemical properties can be easily controlled [7, 8]. Another advantage of enzymatic interesterification over chemical interesterification is the milder processing conditions and possibility of regiospecificity and fatty acid specificity [8]. For the production of nutritionally superior fats, enzymatic interesterification is preferred.

Previous studies have performed interesterification reactions using immobilized enzymes in stirred reactors operating under batch mode [9–11]. However, for industrial-scale production, the use of immobilized enzymes with a packed-bed reactor (a continuous process) is more effective and cost-friendly and allows for reuse of the immobilized lipase [12–14].

Therefore, packed-bed reactor system (packed with immobilized lipase) was developed to continuously produce structured lipids through the interesterification of soybean oil (SO) with fully hydrogenated canola oil (FHCO). Statistical experiment design and analysis were employed to investigate the relationship between the reaction variables (reaction temperature, flow rate, and substrate weight ratio) and response (the conversion degree) and to obtain the optimal conditions for the continuous interesterification through a packed-bed reactor system. Also, by placing the packed-bed reactors in series, the novel continuous process system was established to increase the operation stability of the immobilized lipase.

Materials and methods

Materials

Fully hydrogenated canola oil was a gift from Lotte Samkang Co. Ltd. (Seoul, Korea). SO was purchased from C.J. Cheiljedang Corp. (Seoul, Korea). The fatty acid composition (%, w/w) of SO was 0.1 % myristic, 11.0 % palmitic, 0.1 % palmitoleic, 4.7 % stearic, 22.8 % oleic, 53.6 % linoleic, 6.2 % linolenic, 0.5 % arachidic, 0.2 % gadoleic, 0.5 % behenic, and 0.2 % lignoceric acid. The fatty acid composition of FHCO was as follows (%, w/w): 0.1 % caprylic, 0.2 % capric, 2.0 % lauric, 0.9 % myristic, 9.5 % palmitic, 78.6 % stearic, 4.3 % oleic, 1.8 % linoleic, 0.1 % linolenic, 1.6 % arachidic, 0.4 % behenic, and 0.3 % lignoceric acid. The stearic acids in FHCO exist as triacylglycerol (common name tristearin, 87.6 %); tristearin was not detected in SO. FHCO was used in this study because of the impact of stearic acid on serum cholesterol neutrality. Previous data indicated that myristic and palmitic acid raise serum cholesterol levels, while stearic acid has no effect [15]. Lipozyme TL IM, which is an immobilized lipase preparation from *Thermomyces lanuginosus* supported on silica-granules, was purchased from Novozymes (Bagsværd, Denmark). According to the supplier, a catalytic activity of Lipozyme TL IM is 175 IUN/g. One Interesterification Unit Novo (IUN) is defined as the amount of enzyme that converts 0.01 % (w/w) of tristearin per min at the following batch interesterification conditions: substrate (soybean oil:fully hydrogenated soybean oil = 73:27, w/w %) and 70 °C of reaction temperature. Additionally, the bulk and true densities of Lipoxyme TL IM were 420 and 1,830 kg/m^3, respectively, and its granular size was in the range of 0.3–1.0 mm.

All solvents used were high-performance liquid chromatography (HPLC) grade (J.T. Backer Co., Phillipsburg, NJ, USA). The triacylglycerol standards were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals were analytical reagent grade.

Lipase-catalyzed interesterification on a packed-bed enzyme reactor (PBER)

Fully hydrogenated canola oil and SO blends were subjected to enzymatic interesterification in a single-stage PBER. The PBER was based on a glass column (18 cm long with an internal diameter of 2 cm) with an inlet for the FHCO and SO blend and an outlet for interesterified oil. The PBER was filled with 6 g (1,050 IUN) of Lipozyme TL IM, and the reactor was set to the desired reaction temperature by water circulation through the water jacket surrounding the glass column. The temperature of the reservoir containing the blended oil was kept at 80 °C. Lipase-catalyzed interesterification experiments were performed with FHCO and SO at various proportions. The interesterified oil was continuously pumped out of the reactor outlet into the storage vial at various flow rates. The flow rate (mL/min) of the blended oil was regulated using a peristaltic pump considering the residence time of the blended oil in the reactor. During the continuous run, periodic samples were taken from the reactor outlet for further analysis. A schematic representation of our PBER system is shown in Fig. 1.

Conversion degree determination by triacylglycerol profile analysis

Triacylglycerol profiles were analyzed by reversed-phase HPLC. The interesterified product (15 mg) was dissolved in 10 mL of methylene chloride. This solution was filtered through a membrane filter (pore size, 0.45 μm), and 20 μL of filtrate was injected into the HPLC system. The HPLC

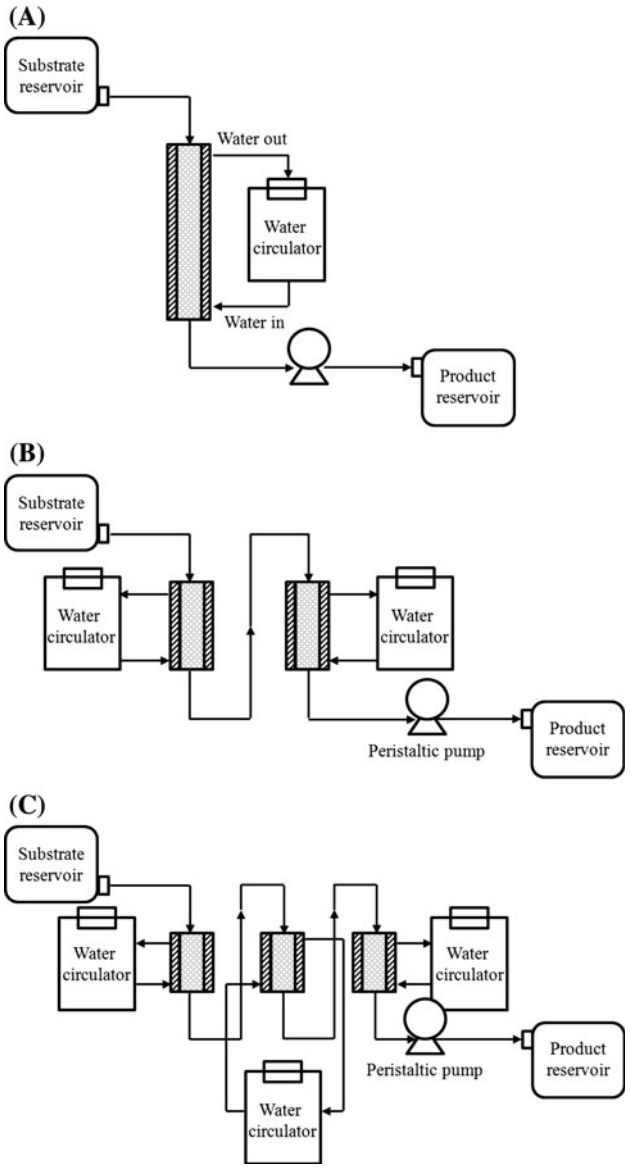


Fig. 1 Schematic diagram of single- (**a**), double- (**b**), and triple-stage (**c**) PBER system for the production of interesterified oils

system was equipped with a Waters 600 controller pump (Waters Corp., Milford, MA, USA) and a Waters 717 plus autosampler (Waters Corp.). The detector was an Alltech ELSD 2000 (Deerfield, MA, USA), which was operated at 80 °C in the drift tube. The nitrogen flow rate was 1.7 L/min. Separation was performed on a Cosmosil $5C_{18}$-AR-II column (4.6 mm I.D. × 250 mm). The column temperature was set at 30 °C. Separation was achieved using a linear gradient elution of the mobile phase A (acetonitrile) and B (methylene chloride), starting from 30 to 50 % B (0–30 min), 50–0 % B (30–35 min), isocratic at 0 % B (35–50 min), 0–30 % B (50–55 min), and then isocratic at 30 % B (55–70 min). The flow rate was 1.0 mL/min. The chromatographic data were recorded and processed using Clarity Lite software (Ver. 2.8.02.648; DataApex, Prague, Czech Republic). In the previous reports, the degree of interesterification was monitored by the consumption or production of a specific triacylglycerol [16–18]. SO does not naturally contain tristearin, and 87 % of the triacylglycerol in FHCO is tristearin. Thus, the change in tristearin content before and after interesterification was used to determine the conversion degree (degree of interesterification). Therefore, the conversion degree (%) was determined according to the following equation [1]:

$$\text{Conversion degree}\,(\%) = \left(1 - \frac{\text{content of tristearin after reaction}}{\text{initial content of tristearin}}\right) \times 100 \qquad (1)$$

Experimental design and statistical analysis

Central composite design (CCD) was used to determine the effects of the substrate weight ratio (FHCO concentration), flow rate, and reaction temperature on the conversion degree in a single-stage PBER system. The five-level and three-factor CCD employed in this study required 20 trials consisting of 2^3 cube points, 6 axial points, and 6 central points (Table 1). We investigated the FHCO concentration (X_1, 34–50 %, w/w), flow rate (X_2, 0.4–2.0 mL/min), and reaction temperature (X_3, 65–85 °C). The examination of these variables was based on a preliminary study. To avoid bias, 20 runs were randomly performed. The conversion of tristearin was considered the response variable. The reaction factors with their levels and responses as actual experimental data are shown in Table 1. All experimental data were analyzed using statistical software (Design-Expert, Ver. 8.0.4.; Stat-Ease, Minneapolis, MN, USA).

Development of multi-stage a PBER system

To determine the operational stability of immobilized lipase in a continuous process, multi-stage PBER system was performed. A schematic representation of the setup is shown in Fig. 1. The double-stage PBER system consisted of two reactors with a glass column (9 cm long with an internal diameter of 2 cm). The height of glass column in the double-stage PBER system was a half of that in the single-stage PBER system half-height glass column. Each PBER was filled with 3 g (525 IUN) of Lipozyme TL IM. The total amount of enzyme in the double-stage PBER system was 6 g (3 g in each reactor). The temperatures in the first and second reactors were maintained at 81.4 and 71.4 °C, respectively. For the triple-stage PBER system, three reactors with glass columns (6 cm long and an internal diameter of 2 cm) were used. The height of the glass column in the triple-stage PBER system was one-third of that in the single-stage PBER system. Each

Table 1 Central composite design consisting of 20 experiments and the response for conversion degree

Run no.	Variables			Conversion degree (%)	
	FHCO (%, w/w) X_1	Flow rate (mL/min) X_2	Temperature (°C) X_3	Actual	Predicted
1	38 (−1)	0.8 (−1)	70 (−1)	80.94 ± 0.51	79.98
2	46 (1)	0.8 (−1)	70 (−1)	76.23 ± 0.35	75.40
3	38 (−1)	1.6 (1)	70 (−1)	64.90 ± 0.29	64.61
4	46 (1)	1.6 (1)	70 (−1)	63.40 ± 0.74	63.52
5	38 (−1)	0.8 (−1)	80 (1)	82.88 ± 0.26	81.65
6	46 (1)	0.8 (−1)	80 (1)	78.10 ± 0.36	77.28
7	38 (−1)	1.6 (1)	80 (1)	67.25 ± 0.24	66.98
8	46 (1)	1.6 (1)	80 (1)	66.25 ± 0.50	66.10
9	34 (−2)	1.2 (0)	75 (0)	76.20 ± 0.75	77.02
10	50 (2)	1.2 (0)	75 (0)	71.28 ± 0.14	71.57
11	42 (0)	0.4 (−2)	75 (0)	84.05 ± 0.44	85.42
12	42 (0)	2.0 (2)	75 (0)	59.12 ± 0.76	58.87
13	42 (0)	1.2 (0)	65 (−2)	67.58 ± 0.42	68.00
14	42 (0)	1.2 (0)	85 (2)	71.57 ± 0.94	72.25
15	42 (0)	1.2 (0)	75 (0)	70.69 ± 0.76	71.20
16	42 (0)	1.2 (0)	75 (0)	71.47 ± 0.67	71.20
17	42 (0)	1.2 (0)	75 (0)	70.81 ± 0.36	71.20
18	42 (0)	1.2 (0)	75 (0)	71.74 ± 0.16	71.20
19	42 (0)	1.2 (0)	75 (0)	71.84 ± 0.20	71.20
20	42 (0)	1.2 (0)	75 (0)	69.52 ± 0.08	71.20

reactor was filled with 2 g (350 IUN) of Lipozyme TL IM. Similar to the single- and double-stage PBER systems, the total amount of enzyme in the triple-stage PBER system was 6 g (2 g in the first, second, and third reactors). The temperatures of the first, second, and third reactors were controlled at 81.4, 71.4, and 61.4 °C, respectively. Following the experiments, samples were collected from the first, second, and third outlet reactors to evaluate the operational stability of the immobilized enzymes and system efficiency.

The reactor volume of single-stage PBER system was 56.52 mL. As described above, because the half and one-third height of reactor (a same internal diameter (2 cm) to single-stage PBER system) was used for double- and triple-stage PBER systems, the total reactor volume of multi-stage PBER system was also 56.52 mL same to the single-stage PBER system. The enzyme bed volume of the dried Lipozyme TL IM was 14.7 mL (14.7 mL for the single-stage PBER system, 7.35 mL for each reactor of double-stage PBER system, and 4.9 mL for each reactor of triple-stage PBER system, respectively), and its void fraction was 0.74. Therefore, the residence time was calculated as $V \times \varepsilon/V_f$, where V is the enzyme bed volume, ε is the void fraction, and V_f is the flow rate.

Analysis of *trans* fats in the interesterified oil

The fatty acid composition was determined by gas chromatography after the preparation of fatty acid methyl esters (FAMEs) according to AOAC Official Method 994.15. Briefly, 25 mg of oil was weighed in a glass tube. Next, 1.5 mL of 0.5 N NaOH in methanol was added and incubated for 5 min at 100 °C to saponify the oil. After incubation, 2 mL of 14 % boron trifluoride in methanol was added and incubated again at 100 °C for 30 min. To stop the reaction and extract the FAMEs, 1 mL of isooctane and 5 mL of saturated NaCl were added to the sample, vortexed for 2 min at room temperature, and centrifuged for 5 min at 80×*g* to separate the organic and aqueous phases. The upper organic layer was filtered through an anhydrous sodium sulfate column, recovered into a vial, and analyzed. The FAMEs were analyzed using an SP-2560 (100 m × 0.25 mm i.d. 20 μm thickness; Supelco, Bellefonte, PA, USA) capillary column in an Agilent 6890 gas chromatograph (Agilent Technology, Wilmington, DE, USA) equipped with a flame ionization detector. The column oven temperature was initially held at 150 °C for 1 min and then programmed to increase at 3 °C/min to a final temperature of 230 °C, at which it was held for approximately 30 min. The temperatures of the injector and detector were 230 and 250 °C, respectively. Injection (1 μL) was performed in split mode at a split ratio of 30:1. Helium was the carrier gas with a flow rate of 1.5 mL/min.

Determination of the solid fat content

Measurement of solid fat content (SFC) was performed using a Bruker Minispec mq20 NMR analyzer (Milton,

Canada), according to AOCS Official Method Cd 16b-93. The NMR tube was filled with 4 g of oil, which was melted by incubation at 80 °C for 30 min. After melting, the NMR tube was equilibrated at 60 °C for 30 min, followed by chilling at 0 °C for 60 min, and then held at 10.0, 21.1, 26.7, 33.3, and 37.8 °C for 30 min of tempering before measuring. The melting, chilling, and holding of the samples were performed in a pre-equilibrated thermostated water bath.

Statistical analysis

All experiments were the presence of mean or mean ± standard deviation (triplicate). Analysis of variance (ANOVA) was conducted, and the mean separations were analyzed by the Duncan's multiple range test ($P < 0.05$).

Results and discussion

Analysis of the central composition experiment

The interesterification conditions, including the FHCO content (X_1), flow rate (X_2), and reaction temperature (X_3), were optimized as independent variables in a single-stage PBER system to maximize the conversion degree. The central composite experimental design and corresponding response values are shown in Table 1. Through statistical analysis, a second-order polynomial model describing the correlation between the conversion degree and the three variables in this experiment was obtained in equation [2], as shown below:

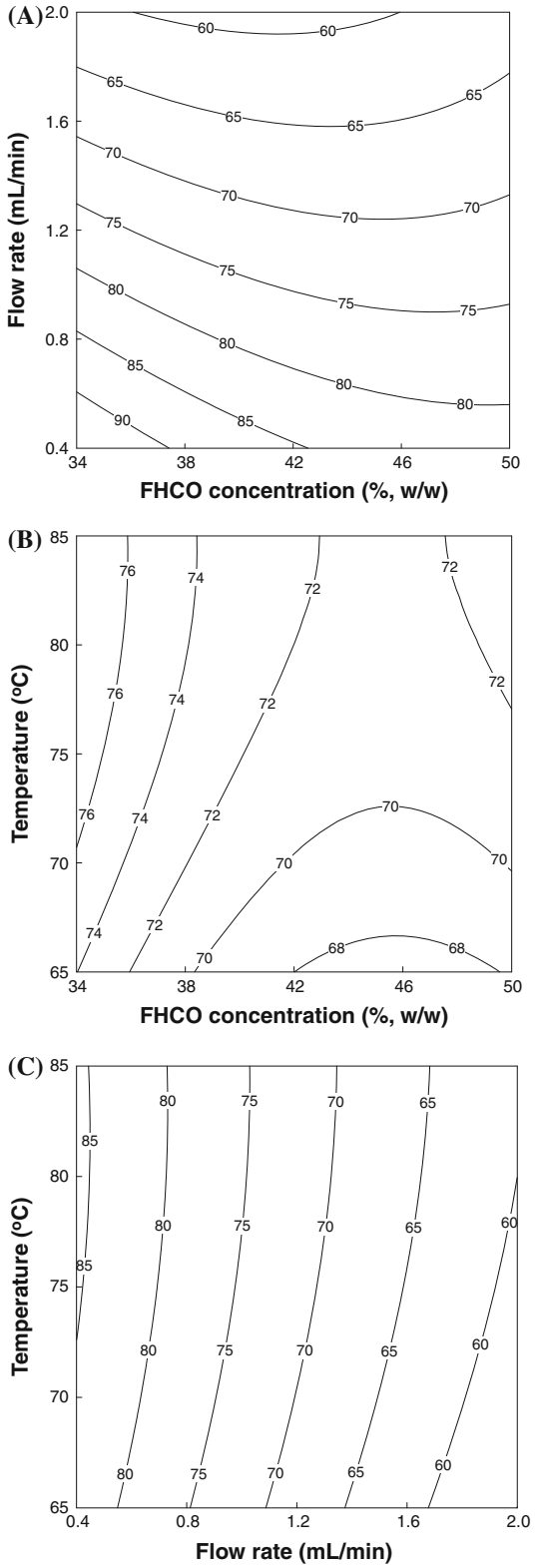
$$\begin{aligned} Y(\text{conversion degree, \%}) &= 160.36438 - 5.25683X_1 - 49.50777X_2 \\ &\quad + 1.60214X_3 + 0.048409X_1^2 + 1.47372X_2^2 \\ &\quad - 0.010685X_3^2 + 0.54505X_1X_2 + 0.00260417X_1X_3 \\ &\quad + 0.086458X_2X_3 \end{aligned} \quad (2)$$

The statistical significance of equation [2] was confirmed by an analysis of variance (Table 2). The effects of variables as linear, quadratic, or interaction coefficients on the response were also evaluated. The coefficient of multiple determinations of the polynomial model (termed R^2) was 0.9857, with no significant lack of fit at $P > 0.05$. This suggests that the fitted model was able to explain 98.57 % of the variability in the response [19]. The results in Table 2 indicate that the model used to fit the response variables was significant ($P < 0.0001$) and adequate to represent the relationship between the responses and independent variables. As shown in Table 2, the FHCO concentration had significant linear and quadratic effects ($P < 0.05$). Flow rate also had a significant linear effect ($P < 0.0001$) but no quadratic effect. Reaction temperature had a significant linear effect ($P < 0.001$) but no quadratic effect. However, none of the independent variables interacted significantly, with the exception of FHCO concentration and flow rate (P values: $X_1X_2 = 0.0430$, $X_1X_3 = 0.8927$, and $X_2X_3 = 0.6558$). Corresponding variables are significant if the P value decreases [20]. A coefficient of variation (CV, 1.48 %) <5 % indicates good experimental precision and reliability, and that the model is reproducible.

Table 2 Analysis of variance of the response surface quadratic model

Source	Sum of square	Degree of freedom	Mean squares	F value	P value	
Model	779.75	9	86.64	76.43	<0.0001	Significant
X_1—FHCO concentration	29.78	1	29.78	26.27	0.0004	
X_2—Flow rate	704.95	1	704.95	621.84	<0.0001	
X_3—Reaction temperature	18.07	1	18.07	15.94	0.0025	
X_1X_2	6.08	1	6.08	5.37	0.0430	
X_2X_3	0.022	1	0.022	0.019	0.8927	
X_2X_3	0.24	1	0.24	0.21	0.6558	
X_1^2	15.08	1	15.08	13.31	0.0045	
X_2^2	1.40	1	1.40	1.23	0.2928	
X_3^2	1.79	1	1.79	1.58	0.2370	
Residual	11.34	10	1.13			
Lack of fit	7.54	5	1.51	1.98	0.2351	Not significant
Pure error	3.80	5	0.76			
Corrected total	791.09	19				

$R^2 = 0.9857$, adj $R^2 = 0.9728$



◀**Fig. 2** Contour plots showing the effects of the independent variables on the conversion degree. **a** Effect of the FHCO concentration (X_1) and flow rate (X_2) on the conversion degree at a reaction temperature of 75 °C. **b** The effect of FHCO concentration (X_1) and reaction temperature (X_3) on the conversion degree at a flow rate of 1.2 mL/min. **c** The effect of flow rate (X_2) and reaction temperature (X_3) on the conversion degree at an FHCO concentration of 42 %

Optimization of interesterification and model validation

Figure 2 shows the effects of the independent variables on the conversion degree in the single-stage PBER system. Figure 2a shows the effect of FHCO concentration and flow rate on the conversion degree at a reaction temperature of 75 °C. When the FHCO concentration and flow rate decreased, the conversion degree increased, indicating an interaction between the FHCO concentration and flow rate. The effect of the FHCO concentration and reaction temperature on the conversion degree at a flow rate of 1.2 mL/min is shown in Fig. 2b. At a high reaction temperature, the change in FHCO concentration had a small effect on the conversion degree (72–76 %). Also, at a low reaction temperature, a small degree of change in the conversion degree (68–74 %) was observed. This suggests that flow rate had a stronger influence on the conversion degree than did the FHCO concentration and/or reaction temperature. Also, it indicates that residence time is an important factor in the conversion degree. Figure 2c shows the effect of flow rate and reaction temperature on the conversion degree at an FHCO concentration of 42 %. When the reaction temperature was constant, the conversion degree increased with decreasing flow rate. Our finding that flow rate is an important variable in a PBER system is consistent with that from a previous study [21].

Based on the obtained model, maximum conversion was achieved at an FHCO concentration of 38.2 %, flow rate of 0.4 mL/min, and a reaction temperature of 81.4 °C. The experiment was performed under these conditions to examine the adequacy of the predicted model. No significant difference between the predicted (89.48 %) and actual (87.09 ± 0.17) conversion degrees was observed, suggesting that the model designed in this experiment was valid.

Operational stability of the immobilized lipase in the multi-stage PBER system

The operational stability of the immobilized enzyme is a very important factor for industrial applications. In general, operational stability depends on various factors, including the reaction temperature and medium, and the linkage of the enzyme to the supporting material [22, 23]. High operational stability of the immobilized enzyme allows for continuous processing, is more effective and economical (because the enzyme can be reused), and is significantly faster. For industrial applications, continuous lipase-catalyzed interesterification requires high operational stability.

For efficient use of the immobilized lipase, a multi-stage PBER system was developed, as shown in Fig. 1. Interesterification was performed in the PBER system for 250 h, and the conversion degree was monitored by determining the change in tristearin content. Interesterification in the single-stage PBER system was performed under optimal conditions (38.2 % FHCO concentration, 0.4 mL/min flow rate, and 81.4 °C reaction temperature) obtained by the predicted model. The double-stage PBER system was performed at 38.2 % FHCO concentration, 0.4 mL/min flow rate, and 81.4 and 71.4 °C reaction temperatures for the first and second reactors, respectively. The triple-stage PBER system was performed at 38.2 % FHCO concentration, 0.4 mL/min flow rate, and 81.4, 71.4, and 61.4 °C reaction temperatures for the first, second, and third reactors, respectively. Reaction temperature is a fundamental factor in lipase-catalyzed interesterification. Generally, when the reaction temperature is elevated, the rate of interesterification increases [24]. However, high reaction temperatures can result in irreversible denaturation of the lipase and a dramatic reduction in the interesterification rate. Lipase immobilization decreased the sensitivity to temperature compared with native lipase when the temperature ranged from 45 to 65 °C. Covalent linkage of the lipase to the supporting material may limit its conformational flexibility [25] and allow the immobilized lipase to maintain an active conformation at high reaction temperatures. As described in "Materials and methods", the temperature of the substrate (blended oil) was kept at 80 °C to prevent solidification. Based on a preliminary study, the reaction temperature was held high to liquefy the blended oil containing FHCO. However, the optimum temperature for maximum conversion was high enough to quickly deactivate the immobilized lipase. Therefore, to increase the operational stability of the immobilized lipase in the continuous process, a temperature gradient multi-stage PBER system was constructed. Figure 3a shows the conversion degrees for single- and multi-stage PBER systems as a function of time. Maximal conversion in the single- and multi-stage PBER systems was observed in the early stages of continuous interesterification, followed by a continuous decrease. In relation to the conversion degrees, the operational time (half-life, $t_{1/2}$) to reach 50 % residual activity was 121.6, 165.0, and 198.0 h for the single-, double-, and triple-stage PBER systems, respectively. The higher operational stability of the immobilized lipase in the multi-stage PBER system compared with the single-stage PBER system was likely caused by decreased deactivation of the lipase resulting from the low reaction temperature in the second and/or third reactor. The melting point of the interesterified oil through the first reactor could be lower than that of the blended oil due to the rearrangement of fatty acids by interesterification. Therefore, having the second and/or third reactor at a high temperature (approximately 80 °C) to keep the substrate in the liquid state was unnecessary, indicating that the second and/or third reactor could be run at a lower temperature without any issues caused by oil solidification. Compared with the single-stage PBER system, the multi-stage PBER system showed improved operational stability.

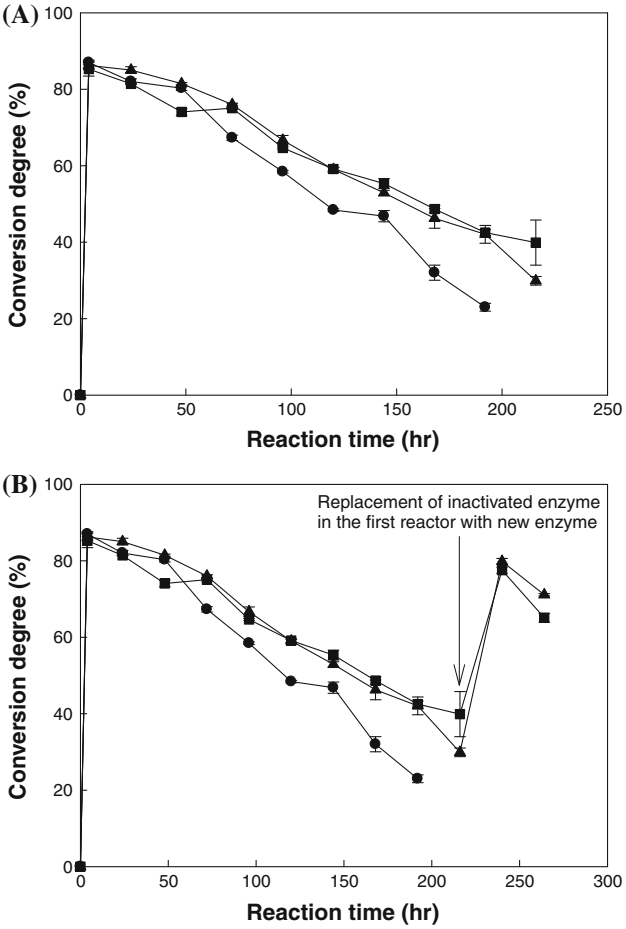


Fig. 3 Production of the interesterified oils through single-, double-, and triple-stage PBER system. **a** Gross conversion degree by lipase-catalyzed interesterification in single- (*filled circle*), double- (*filled triangle*), and triple-stage (*filled square*) PBER systems at 38.2 % FHCO in blended oil at a 0.4 mL/min flow rate. **b** Effect of immobilized lipase replacement in the first reactor on the gross conversion of tristearin in our single- (*filled circle*), double- (*filled triangle*), and triple-stage (*filled square*) PBER systems at 38.2 % FHCO in blended oil at a 0.4 mL/min flow rate. The reaction temperatures used in the first, second, and third reactors are given in "Materials and methods"

When reactors are operated in series under the same conditions, the first reactor typically shows the lowest enzyme activity while the last reactor shows the highest enzyme activity [26]. This may be because in a continuous process with several reactors in series, components that negatively affect the enzyme are eliminated by absorption in the first reactor where the enzyme is rapidly deactivated.

Thus, the first reactor protects the level of enzyme activity in the downstream reactors. However, this phenomenon can also be explained by the different operational temperatures in each reactor. In our multi-stage PBER system, the reaction temperature in the first reactor was the highest at 81.4 °C. According to the immobilized lipase supplier, its optimal temperature is approximately 70 °C. This suggests that the first reactor temperature (81.4 °C) was somewhat high for the immobilized lipase. Control of the first reactor at a higher temperature could prevent solidification of the oil mixture during interesterification, although the operational stability of the enzyme would be reduced. Figure 4 shows the conversion degree in each reactor for the double- and triple-stage PBER systems. In the double- and triple-stage PBER systems, the immobilized lipase in the first reactor lost its activity after 216 and 144 h (Fig. 4), respectively. This difference could be attributed to the different enzyme concentrations in the first reactor in the double- and triple-stage PBER systems. The conversion of tristearin decreased steadily with time in the second reactors of the double- and triple-stage PBER systems (Fig. 4b, d). In the third reactor of the triple-stage PBER system, maximum tristearin conversion (approximately 40 %) was observed at 72 h, followed by a stationary phase lasting another 72 h (Fig. 4c). After the 144-h reaction, the conversion of tristearin decreased.

As shown in Fig. 3a, in all PBER systems, there was a gradual decline in enzymatic activity. This inactivation of the enzyme during reactor operation could be a major issue for maintenance of the continuous process. Changes in the flow rate and reaction temperature, as well as enzyme replacement, may prevent enzyme decay for prolonged period operation [27]. Of these solutions, enzyme replacement is the most straightforward. Figure 3b shows the effect of enzyme replacement in the first reactor on the gross conversion degree. When the immobilized lipase in the first reactor was replaced, the gross conversion degree increased sharply to approximately 80 %. In our experiment, as the number of reactors increased, each reactor contained smaller amounts of immobilized lipase (3 g in each reactor for the double-stage PBER system and 2 g in each reactor for the triple-stage PBER system). This indicates that the operational stability of the immobilized lipase and conversion degree increased significantly in the multi-stage PBER system with different reactor temperatures.

Compared with single-stage PBER system, in economic point of view, multi-stage PBER system could have many advantages even though all serially connected reactors are operated at the same reaction temperature. Because the enzyme activity in the downstream reactors is still enough, the stable operation with longer life time could be achieved by only replacement of enzyme in the first reactor. In

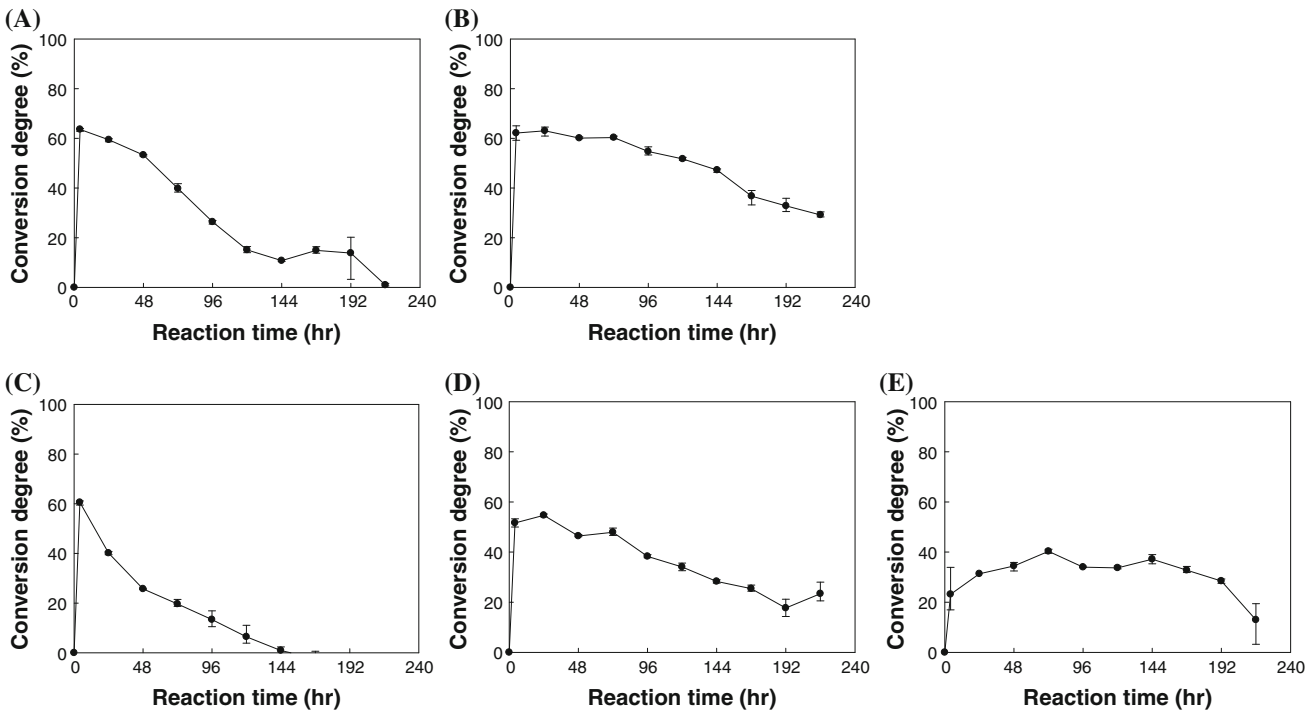


Fig. 4 Conversion degrees of the first (**a**, **c**), second (**b**, **d**), and third (**e**) reactors in the double- (**a**, **b**) and triple-stage (**c**–**e**) PBER system at 38.2 % FHCO in blended oil at a 0.4 mL/min flow rate. The operational temperatures for first, second, and third reactors were 81.4, 71.4, and 61.4 °C, respectively

results, food industry could continuously produce the interesterifided lipid with the low cost. Furthermore, when each reactor in multi-stage PBER system is operated at the different temperature, especially downstream reactors at the lower temperature than the first reactor, food industry should save energy due to the low temperature heat inputs. In summary, by operating the multi-stage PBER system at the different reaction temperatures for each reactor, food industry could produce highly valued interesterified lipid with low cost.

Interesterification rearranges fatty acids in triacylglycerol molecules, but this reaction did not form *trans* fatty acids. In this study, the content of *trans* fatty acids was 0.08 g in 100 g of blended oil and 0.09, 0.09, and 0.07 g in 100 g of interesterified oil through the single-, double-, and triple-stage PBER systems, respectively. This observation is consistent with that in the previous studies [28, 29]. "*Trans*-fat free" is included on the nutrition label if the *trans* fat content is less than 0.5 g per serving in the United States. In Europe, "*trans* fat free" can be declared on the nutrition label when the *trans* fat content is less than 2 % of the total fat in food, following Danish legislation. In comparison, in Korea, to claim that a product is "*trans* fat free", it must contain less than 0.2 g of *trans* fat per serving.

SFC of interesterified oils

The SFC is the percentage of lipid that is solid at various temperatures. This value is required to understand the physical properties of solid fats such as shortening. Figure 5 shows the SFC of the interesterified oils in single-, double-, and triple-stage PBER systems. Interesterification was performed under the optimized conditions established using single-stage PBER system through response surface methodology. The blended oil had a high SFC at all temperatures from 10 to 37.8 °C. The SFC for all samples declined linearly with increases in temperature, and the interesterified oils tended to have lower SFC values than the blended oil at all measured temperatures. All of the interesterified oils had similar SFC profiles, regardless of the number of reactors. These results could be due to rearrangement of the fatty acids in the triacylglycerols during interesterification, followed by the formation of altered triacylglycerols with lower melting points than those in the blended oil. A previous study reported that interesterification altered polymorphic forms [10]. The polymorphic forms in the blended oil ($\beta' < \beta$ form) changed to more β' polymorphic forms in the interesterified

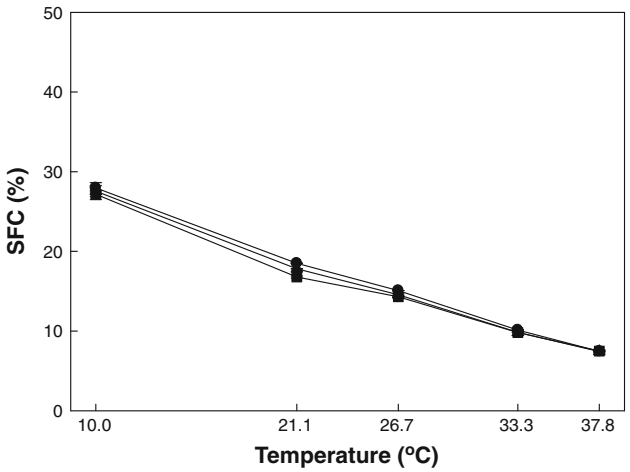


Fig. 5 SFC (%) of the interesterified oils in the single- (*filled circle*), double- (*filled triangle*), and triple-stage (*filled square*) PBER systems

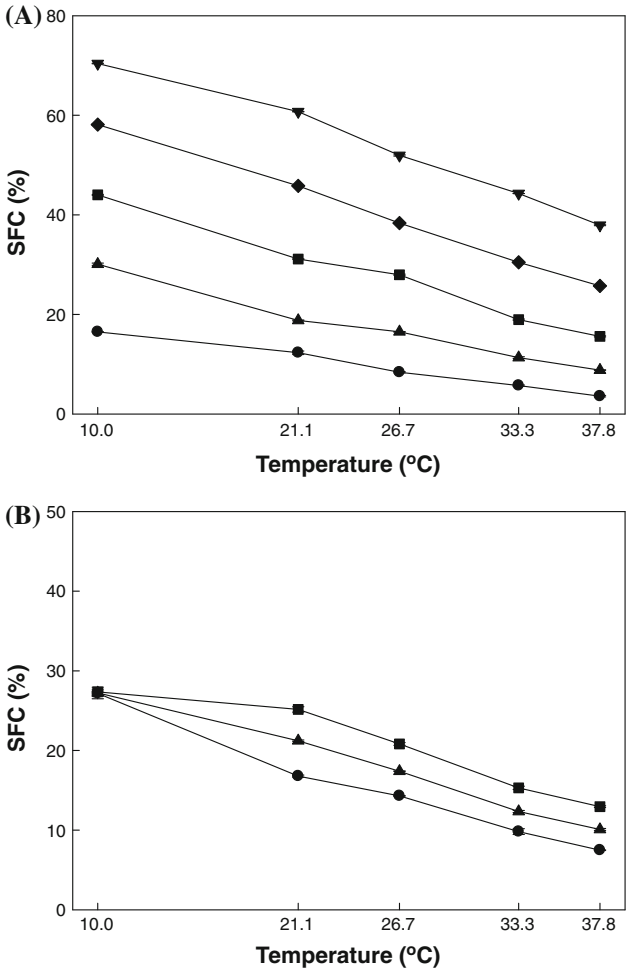


Fig. 6 Effect of the PBER parameters for continuous interesterification on the SFC profiles of the interesterified oils. **a** Triple-stage PBER system at a flow rate of 0.4 mL/min and a reaction temperature of 81.4, 71.4, and 61.4 °C for the first, second, and third reactors, and at a FHCO concentration of 30 (*filled circle*), 40 (*filled triangle*), 50 (*filled square*), 60 (*filled diamond*), and 70 (*inverted triangle*) %, respectively. **b** Triple-stage PBER system at a FHCO concentration of 38.2 %with a reaction temperature of 81.4, 71.4, and 61.4 °C in the first, second, and third reactors, and at a flow rate of 0.4 (*filled circle*), 0.8 (*filled triangle*), and 1.2 (*filled square*) mL/min, respectively

oil ($\beta' > \beta$ form). Since the β form has a higher melting point than the β' form, the altered polymorphic form could result in lower SFC values in the interesterified oils. It was found out that interesterification altered the SFC value of the blended oil by the changing polymorphic form. Regarding the above results, the production of interesterified oils with various SFC profiles is possible by changing interesterification conditions in the PBER system. The interesterified oils produced under our modified interesterification conditions showed various SFC profiles (Fig. 6).

Conclusions

The optimal conditions for continuous interesterification through PBER system were established. The optimal conditions for tristearin conversion are as follows: 38.2 % FHCO concentration, 0.4 mL/min flow rate, and 81.4 °C reaction temperature. The operational stability of the immobilized lipase in the multi-stage PBER system was. The production of oils with various melting range was possible through PBER system by adjusting the interesterification conditions, and these oils could be suitable for use in a bakery, margarine, or for general cooking purposes. This study demonstrates the potential for the industrial production of structured lipids using a PBER system with immobilized lipase.

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