

Structured and Specialty Lipids in Continuous Packed Column Reactors: Comparison of Production Using One and Two Enzyme Beds

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Received: 3 June 2009 / Revised: 8 October 2009 / Accepted: 2 November 2009
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Abstract In this study, speciality lipids based on fish oil (FO) and capric acid (CA) were produced in packed bed bioreactors using immobilized Lipozyme IM from *Rhizomucor miehei* in a solvent-free environment. Our goal was to compare the product quality and yield among reactors consisting of one or two enzyme columns. Response surface methodology (RSM) was used to optimize process variables for maximum incorporation (Inc) of CA for each reactor configuration. The studied process variables were substrate molar ratio (1:1–3:1 CA/FO), temperature (35–55 °C) and flow rate (0.5–1.5 mL/min). All experiments were conducted based on a face-centered cube design. The maximum predicted Inc of CA into FO (31.7 mol%) using one column was obtained when substrate molar ratio, temperature and flow rate of substrates were 2.70:1 (CA/FO), 55 °C and 0.5 mL/min. The corresponding optimal Inc of CA into FO (22.7 mol%) using two columns was predicted at 2.95:1 (CA/FO), 55 °C and 0.86 mL/min. Analysis of variance (ANOVA) showed that Inc of CA into the one-bed design was significantly influenced by all experimental conditions, with substrate molar ratio having the greatest impact. In the two-bed design, only temperature and flow rate had an effect; the molar ratio of substrates was not significant. Coefficients of determination were low for both designs, indicating a poor fit of our data to the model. However, the main purpose of this study was to assess the effect of process factors on Inc of CA into FO, rather than generate a model useful for prediction of responses under conditions not examined with this

design. Therefore, the significant ANOVA results are much more important, stressing the real relationship between parameters and response, than the low coefficients of determination.

Keywords Acidolysis · Structured lipids · Capric acid (10:0) · Response surface methodology (RSM) · Packed bed bioreactor · Immobilized lipase *Rhizomucor miehei*

Introduction

Specialty lipids include a wide range of products amongst which structured lipids are a main class. Structured lipids (SL) are triacylglycerols (TAG) or phospholipids (PL) in which fatty acids (FA) are placed in specific locations in the glycerol backbone and are produced using a chemical or enzymatic process. Much attention is being paid to SL due to their potential biological functions and nutritional benefits. Lipids can be restructured to meet essential FA requirements or to incorporate specific FA of interest. SL may also offer the most efficient means of delivering target FA for nutritive or therapeutic purposes. For instance, a SL containing a mixture of medium-chain FA (MCFA; 6–12 carbon atoms) for rapid energy release, and long-chain polyunsaturated FA (LCPUFA, >12 carbon atoms), serving as a source of essential FA, could be useful in the alleviation of specific diseases and metabolic conditions [1].

Strategies for lipid modification include genetic engineering of oilseed crops, production of concentrated oils containing high levels of polyunsaturated or other types of FA, and lipase- or chemically-assisted interesterification reactions [2]. Depending on the type of substrate available, chemical or enzymatic reactions can be used for the

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synthesis of SL, including direct esterification (reaction of FA and glycerol), acidolysis (transfer of an acyl group between an acid and ester), and alcoholysis (exchange of an alkoxy group between an alcohol and an ester). However, the usual methods cited in the literature for production of SL are based on reactions between two TAG molecules or between a TAG and a FA. Although chemical synthesis of SL is cheap and easy to scale up, it suffers many drawbacks, including a lack of specificity and control over the positional distribution of FA in the final product. An alternative to chemical production of SL is a bioconversion process using lipases. Such a process produces fats or oils with a defined structure because it incorporates a specific FA at a specific position of the glycerol moiety. It requires only mild experimental conditions without potential for side reactions, and offers a reduction in energy consumption, reduced heat damage to reactants, and easy purification of products [2]. However, bioconversion of lipids with lipases is more expensive than chemical methods. Therefore, immobilization of lipases on suitable supports is desirable as it allows easy recovery and reuse of the enzymes, resulting in reduced cost of production. Immobilized lipase from *Rhizomucor miehei* (Lipozyme IM) is a good biocatalyst because it has a *sn*-1,3 stereospecificity, and a weak activity toward long-chain n-3 FA especially docosahexaenoic acid (DHA) [3, 4]. Because of these features, Lipozyme IM may be particularly suited for the synthesis of SL that incorporate maximum MCFA, and at the same time maintain as much PUFA as possible.

Oils from fish and other marine creatures can be restructured or altered to incorporate MCFA for certain desired nutritional characteristics. Over the past decade several research groups have successfully used packed (or fixed) bed bioreactors (PBR) in the lipase-assisted synthesis of SL [5–18]. For instance, Fomuso and Akoh [15] produced optimal solvent-free SL at a substrate flow rate of 1 mL/min, 2.7 h, 60 °C, and 5:1 (8:0/olive oil). The formation of SL was significantly correlated to the flow rate and reached equilibrium at 30–40 min at 60 °C using immobilized lipase from *Thermomyces lanuginosa*. Similarly, Xu et al. [10, 12, 13] reported that the extent of reaction progress in the formation of SL was related to the residence time. *R. miehei* lipase has also been used to catalyze the Inc of 8:0 into vegetable oils. For example, the Inc of 8:0 into sunflower oil was found to increase steadily as the molar ratio of 8:0 to sunflower oil increased from 1:1 to 8:1 [6]. Xu et al. [10] also demonstrated the production of SL based on rapeseed oil and capric acid (CA) in a continuous PBR using *R. miehei* lipase. Incorporation of CA (66.2%) into the terminal positions of the resultant SL was obtained at 6:1 (CA/oil), and 60 °C, over 151 min.

Nonetheless, the previously mentioned studies did not investigate ways to produce SL in consistent amounts and

quality or to expand production through the incorporation of more than one enzyme bed into the bioreactors. PBR are increasingly attracting attention because of the potential applications in industrial lipid modification. PBR are used for most large-scale enzymatic processes because of high effectiveness, low capital investment, ease of construction, potential for continuous operation, and ease of maintenance [8]. Here, we investigate the feasibility of producing SL based on fish oil, known as MEG-3, and CA in PBR of different designs. The specific objectives were: [1] to construct PBR with one and two enzyme beds; [2] to investigate the effects of flow rate, reaction temperature and substrate molar ratio on CA Inc into FO; and [3] to optimize the process variables using response surface methodology. We hypothesized that incorporation of a second column into the PBR would increase the residence time and hence time available for the enzyme to interact with the substrates. As a result, we expected %Inc of CA into FO to be higher using the two-bed bioreactor.

Materials and Methods

Materials

Fish oil, known as MEG-3 was kindly provided by Ascenta Health (Dartmouth, NS, Canada). The major FA (mol% of total FA identified) present in the MEG-3 were: 14:0 (8.7 mol%), 16:0 (17.6 mol%), 16:1n-7 (9.0 mol%), 16:4n-1 (2.6 mol%), 18:0 (3.1 mol%), 18:1n-9 (8.6 mol%), 18:1n-7 (2.7 mol%), 18:4n-3 (2.5 mol%), 20:5n-3 (16.2 mol%), 22:5n-3 (1.6 mol%), 22:6n-3 (9.1 mol%). Capric acid (CA; decanoic acid; $\geq 98.0\%$) and a commercial *sn*-1,3-specific lipase from *R. miehei*, immobilized on weakly basic macroporous anion exchange resin were purchased from Sigma Chemical Co. (St. Louis, MO). FA methyl esters (FAME) standards were purchased from Nu-Chek Prep (Elysian, MN).

Apparatus

The apparatus (see Fig. 1) consisted of one or two enzyme beds, product and substrate containers, a water bath, and peristaltic pumps. The columns had a 25-mm inner diameter (id), a 33.5-mm outer diameter (od), and a length of 162 mm. The substrate container (feeding tank) was a 1-L dark glass vessel. Four holes were drilled through the lid: a 10 mm diameter opening in the center of the lid for the mixing shaft, and three 8-mm diameter holes for the air outlet, nitrogen inlet, and substrates outlet. A steel mixing shaft of 8 mm diameter and a 40-mm length was installed through the center of the lid. The mixing shaft was driven by a variable speed electric motor. This reservoir was

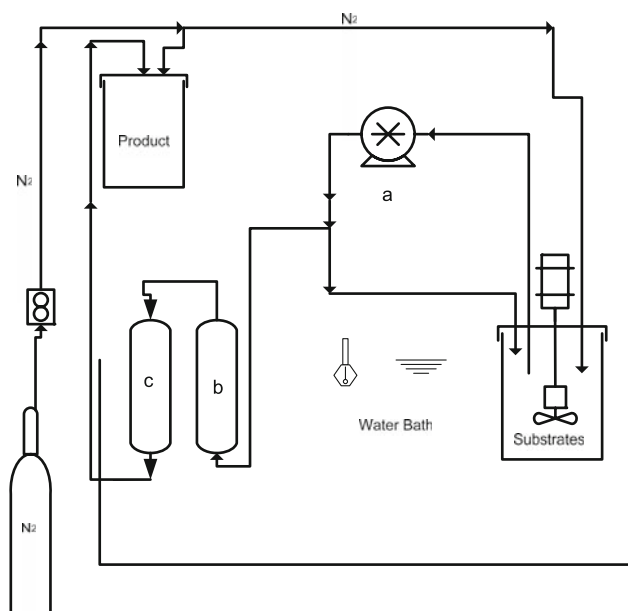


Fig. 1 Schematic diagram of the two-bed bioreactor. *a* peristaltic Master Flex pumps, *b* and *c* enzyme beds packed with immobilized lipase

nitrogen protected. The reactants container was placed in a water bath. The product container was a 50-mL graduated cylinder fitted with a two-holed stopper. The nitrogen gas was supplied to the substrates and product tanks through flow meters with high-resolution valves using Norprene tubing (5 mm diameter). The nitrogen gas was introduced into the feeding and collecting containers from the top of these containers.

Experimental Protocol

Packing of the Column and Determination of the Bed Void Fraction

The water activity of immobilized lipase was determined (0.196) prior to loading onto the bed. The water activity was measured using a water activity meter (Aqua Lab Model CX-2). The immobilized enzyme (24 g, dry basis) was then loaded directly onto the column. The upper and lower ends of the bed were layered each with glass wool (1 and 2 cm in thickness, respectively) to prevent pressure drop inside the enzyme column. The same enzyme packing was used for all experiments.

Following the Method of Vikbjerg et al. [8] hexane was fed into the packed column at ambient temperature. The bed was shaken gently to remove bubbles in the liquid until the enzyme bed was completely filled with hexane. The column mass was taken before and after filling the bed with hexane. The total volume of the enzyme bed (V_t) was then

calculated from the diameter of the column and length of the bed ($=76.0$ mL); the void volume (V_v) was calculated using the density and mass of hexane and was found to be 55.0 mL. The void fraction was then calculated (0.724) using $\varepsilon = V_v/V_t$. After determination of dead volume of the bed, hexane was eluted from the bottom of the column. The upper and lower end of the column was attached to the reactor and nitrogen was passed through to remove air.

The PBR was operated in continuous mode by directing the column eluent to the product container. The reaction mixture (FO and CA) was solvent-free and no water was added. The substrates reservoir was attached to a nitrogen source, a mixing motor, and a peristaltic pump. The substrate mixture was then placed in the substrate container and stirred at 100 rpm using an impeller driven by a mixing motor. The temperature of the PBR was maintained by circulating hot water at specific temperatures (35–55 °C). When an experiment was started, prepared substrates were pumped into the enzyme bed at the set variables for conditioning over-night. The recycling of substrates in Fig. 1 has been made for practical reasons in order to obtain the desired flow rate. The first 1–2 V_v eluted from the beds were discarded before collecting samples for analysis. During each experiment, the temperature of the system and the substrates flow rate was closely monitored. When the reaction was terminated, the product was drained from the product container and thoroughly mixed and a portion of it was transferred to a small vial, flushed with nitrogen, and stored at -20 °C for further analysis. The entire reaction system (packed bed, containers, and connecting tubes) was washed by circulating 100–200 mL of hexane. Then nitrogen gas was flushed into the bed, thus preparing the reactor for another reaction.

Experimental Design

The residence time of the substrates in the reactor was calculated as described by Xu et al. [13].

$$\text{Residence time (min)} = V_t \cdot \varepsilon / F_R$$

where V_t is the total enzyme bed volume (76.0 mL), ε is the void fraction, and F_R is the volume flow rate (mL/min). The F_R of substrates in the one-bed design was varied from 0.5 to 1.5 mL/min which is equivalent to a residence time of 110–37 min, respectively. The same F_R of the substrates was used in the two-bed bioreactor and the corresponding residence time was 220–74 min, respectively. Each experiment was conducted in triplicate.

A three-factor and three-level model based on a face-centered cube design was selected for this study [5, 19]. This design typically uses 17 design points with 8 factorial and 6 axial points, and three replicates of the center point. We chose to incorporate more replication into the design

Table 1 Face-centered cube design arrangement and response for the analysis of fish oil (MEG-3) with capric acid (10:0) using single and dual bed reactors with immobilized lipase

Molar ratio (CA/FO)	Temperature (°C)	Flow rate (mL/min)	Response %CA Inc (mol%)	
			One bed	Two beds
1:1(-1)	35(-1)	1.5(+1)	8.5 ± 0.5	11.4 ± 2.1
3:1(+1)	35(-1)	0.5(-1)	22.5 ± 2.0	12.9 ± 1.4
1:1(-1)	35(-1)	0.5(-1)	11.6 ± 1.2	18.8 ± 0.4
2:1(0)	35(-1)	1.0(0)	15.1 ± 0.9	20.9 ± 1.0
3:1(+1)	35(-1)	1.5(+1)	12.0 ± 2.9	6.8 ± 1.4
2:1(0)	45(0)	1.5(+1)	17.1 ± 5.2	10.2 ± 1.1
3:1(+1)	55(+1)	0.5(-1)	31.8 ± 7.0	18.0 ± 0.8
3:1(+1)	55(+1)	1.5(+1)	28.6 ± 1.8	12.9 ± 1.4
2:1(0)	45(0)	1.0(0)	21.7 ± 1.3	10.8 ± 0.5
1:1(-1)	45(0)	1.0(0)	14.3 ± 0.5	11.0 ± 0.5
3:1(+1)	45(0)	1.0(0)	13.1 ± 1.3	24.2 ± 6.9
2:1(0)	55(+1)	1.0(0)	22.5 ± 0.8	22.2 ± 1.2
2:1(0)	45(0)	0.5(-1)	27.5 ± 1.1	15.3 ± 0.7
1:1(-1)	55(+1)	0.5(-1)	17.0 ± 1.1	22.3 ± 0.3
1:1(-1)	55(+1)	1.5(+1)	17.5 ± 0.7	12.3 ± 1.8

by using three replicates of all factorial and axial points as well, yielding a total of 45 measurements of Inc. The independent factors were coded for an experimental design. The center point for each independent variable level was given a code of zero. The highest and lowest levels for each independent factor were coded plus or minus one, respectively, for this three level design. To avoid bias, 45 runs were performed in a totally random order to minimize the impact of unexplained variability in the observed response (see Table 1 for combinations of factors tested). The independent variables or factors investigated were molar ratio of substrates (MR; 1:1 to 3:1 moles of CA/FO; X_1), reaction temperature (T ; 35–55 °C; X_2) and F_R of substrates (0.5–1.5 mL/min; X_3). Response or dependent variables studied were: Y_1 for one bed (CA%), and Y_2 for two columns reactor (CA%). To monitor process stability, replicates of one design point (i.e., 0.5 mL/min F_R , 35 °C, 1:1 MR) were performed at the beginning, middle and end of the 45 experiments.

The second-order polynomial model used for optimization of CA Inc into fish oil (Y) was:

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

Where, β_0 , β_i , β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are the independent variables. Data were analyzed using the Design Expert version 7.1.6 software from Stat Ease Inc. (Minneapolis, MN, USA). Response surfaces were obtained using the fitted model, by keeping the least effective independent variable at a center value while changing the other two.

Isolation of Acylglycerols after Acidolysis and Analysis of FAME

Purification of acylglycerols after acidolysis reactions was performed according to Hamam and Shahidi [19]. Briefly, free FA (FFA) in the sample reaction mixture were neutralized with NaOH using phenolphthalein as indicator and the acylglycerol fraction was recovered with hexane. FA profiles of products were determined following their conversion to methyl esters. FAME were prepared and analyzed in triplicate as described in Budge et al. [20] using sulphuric acid in methanol. The FAME were analyzed using a Perkin Elmer Autosystem Capillary GC with a flame ionization detector using a flexible fused silica column (30 m × 0.25 mm id) coated with 50% cyanopropyl polysiloxane (0.25 μm film thickness; J and W DB-23; Agilent, Palo Alto, CA). Details of the GC analysis, including temperature program, can be found in Budge et al. [20]. Response factors for FAME were applied, and FAME were reported as mole percent of total FAME identified.

Results and Discussion

Response Surface Methodology

RSM is a statistical design that enables one to determine optimal conditions for enzymatically-catalyzed reactions by conducting a minimum number of experiments. The results of traditional one-variable-at-a-time analyses do not indicate real changes in the environment as they disregard

interactions among variables. RSM can describe simultaneous influences more fully and help in a more precise optimization of variables that affect the process. A design based on a face-centered cube was employed in this study. This design was selected over others, such as a rotatable design, because it uses only three levels of each variable, whereas other central composite designs would need five levels of each [5]. Having three levels instead of five is advantageous because it decreases the time of preparation and increases the economy of the process.

RSM Results for the Single Bed Design

Multiple regression coefficients were calculated using a least squares procedure to predict the second-order polynomial model for CA Inc into FO for each bioreactor configuration using the experimental data (Table 2). T and MR of reactants had a positive effect on %CA Inc (Table 2; Fig. 2), while F_R (Fig. 3) had a negative effect. Furthermore, the quadratic term of F_R^2 had a significant positive impact on %CA Inc into FO, while the quadratic term of MR^2 had a significant negative effect. There were no significant interactions of experimental parameters. The best way to examine the links between the response, parameters, and interactions that existed in the one-bed model is to analyze the surface response (Figs. 2, 3). Higher T and MR resulted in higher Inc of CA into FO (Fig. 2) while lower F_R (longer R_T) and higher T gave the greatest %Inc of CA (Fig. 3). Relatively large coefficients of determination ($R^2 = 0.79$) indicated an adequate fit of the model to the data. Optimal conditions were calculated using the optimizer function of the Design Expert and

approximate values can easily be deduced through visual inspection of the response surfaces (Figs. 2, 3). Maximum Inc of CA into FO (31.7 mol%) using one column was predicted when MR, T and F_R were 2.70:1 (CA/FO), 55 °C and 0.5 mL/min ($R_T = 110$ min), respectively.

A reactor temperature of 35 °C was selected as the starting point for the acidolysis of FO and CA because CA has a melting point of 31–32 °C; no reaction was conducted above 55 °C because the optimal temperature for most lipase falls within the range 30–62 °C [15]. Therefore, the extent to which the temperature can be increased is restricted by the fact that extreme temperatures will totally and irreversibly denature the lipase. The effect of temperature on reaction rate of an enzyme-catalyzed reaction, prior to denaturation temperature, is well-established. In general, a 10 °C increase in temperature results in a doubling of the reaction rate and hence a higher Inc of CA. Higher temperatures are thought to increase enzyme activity and improve contact between the substrates and enzyme as the viscosity decreases. Using a single enzyme bed, Inc of CA into FO did increase with an increase in temperature. Others have found similar results. For example, Mu et al. [6] found maximum Inc (13–40%) of 8:0 into vegetable oils at a temperature of 60 °C. The rate of 1,3-diacylglycerol production by esterification of FA and glycerol was also found to increase with an increase in temperature up to 50 °C in a PBR under vacuum conditions [14]. Similarly, Xu et al. [11] found that Inc of 8:0 and the content of di-incorporated SL were increased by approximately 20% with a temperature increase from 40 to 70 °C. It seems clear that increasing temperature results in increased incorporation. However, the influence of

Table 2 Regression coefficients of the predicted quadratic polynomial model for responses (Y) for one- and two-bed designs

Variables ^a	Fish oil, %CA using a one-bed reactor (Y_1)		Fish oil, %CA using a two-bed reactor (Y_2)	
	Coefficients	p values	Coefficients	p values
Intercept	19.12		16.43	
Linear				
MR	3.90	<0.0001	-0.16	0.8392
T	4.76	<0.0001	1.72	0.0321
F_R	-2.67	0.0002	-3.36	0.0001
Quadratic				
MR^2	-4.78	0.0007	-0.23	0.8783
T^2	0.35	0.7869	3.72	0.0193
F_R^2	3.82	0.0051	-5.08	0.0020
Interactions				
$MR \times T$	1.44	0.0546	0.80	0.3595
$MR \times F_R$	-1.39	0.0635	0.70	0.4188
$T \times F_R$	1.36	0.0688	-0.15	0.8611
R^2	0.79		0.54	
Model		<0.0001		0.0007

^a Coefficients refer to the general model
 R^2 coefficient of determination

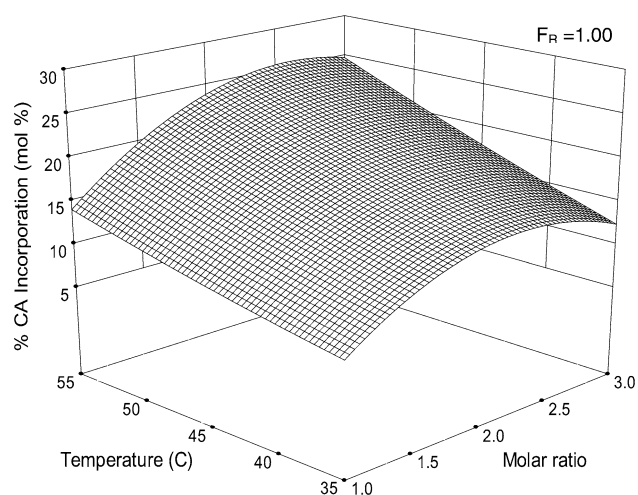


Fig. 2 Three-dimensional plot demonstrating the effects of substrates molar ratio and reaction temperature on the predicted CA incorporation (mol%) into fish oil using the one-bed bioreactor at a constant flow rate of 1 mL/min

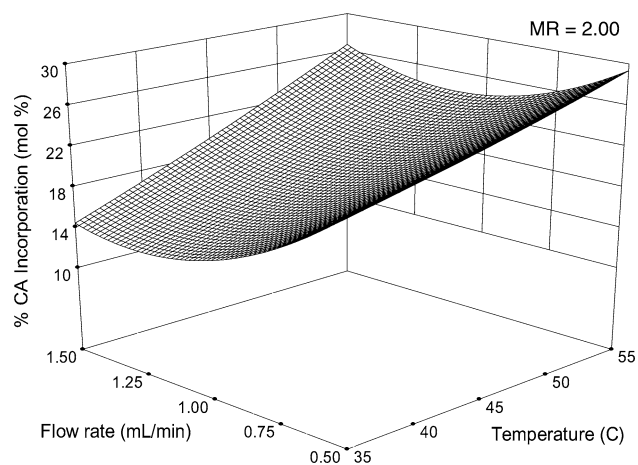


Fig. 3 Three-dimensional plot demonstrating the effects of reaction temperature and substrates flow rate on the predicted CA incorporation (mol %) into fish oil using the one-bed bioreactor at a constant molar ratio of 2:1

temperature on the rates of other reactions should be considered. For instance, higher temperatures will increase the rate of lipid oxidation, especially for polyunsaturated FA (PUFA). Therefore, the highest temperature may not always be optimal for preserving lipid quality and temperatures between 50 and 60 °C are normally recommended for FO containing high levels of PUFA.

MR plays a double role in enzymatic reactions [9]. Higher ratios of CA (as FFA) to TAG shift the reaction equilibrium to favor products and raise the theoretical maximal Inc and product yields. On the other hand, increasing concentrations of FFA cleaved from the TAG molecules in making SL increase the inhibition effect and, hence, lead to longer reaction times to reach completion

[9]. With the single-bed reactor, increasing MR from 1:1 to 3:1 (CA/FO) resulted in a corresponding increase in %Inc of CA into FO. This agrees with a number of other studies (e.g. 6, 12) and is the anticipated result for a reaction that has not reached equilibrium. Higher MR (>3:1) would be expected to result in further increases in %Inc but we chose to use a maximum MR of 3:1 for two general reasons. First, when considering an industrial process, the choice of reactants MR relates to the processing cost and possible difficulties in separating the released FFA from the products. MR of 3:1 was selected because in large scale commercial production higher ratios of substrates would both increase the capital investment since CA is expensive and would also require costly separation of large amounts of unreacted CA from the final product by molecular distillation. Secondly, there is evidence that there is only a slight increase in %Inc with high MR. For instance, Xu et al. [11] reported that increasing MR from 1:1 to 7:1 did increase the Inc of 8:0 into canola oil, but above a MR of 3:1, the change in Inc was not significant. Similarly, Fomuso and Akoh [15] produced optimal Inc of 8:0 into olive oil at MR of 5:1 (8:0/olive oil) but the Inc at 5:1 was only slightly greater than that at 3:1. Thus, little benefit has been seen in increasing our MR above 3:1.

RSM Results for the Dual Bed Design

The degree of Inc of CA into FO using the two-bed model was significantly ($p < 0.05$) affected by linear and second-order terms of T and F_R (Table 2; Fig. 4). Positive effects were observed for the linear and quadratic terms of T , while F_R had a consistent negative effect on Inc for both linear and quadratic terms. In contrast to the single-bed reactor, linear and quadratic terms of MR had no effect on Inc of CA. For instance, in Fig. 5, it is clear that Inc of CA is independent of MR. There were also no significant interactions of experimental variables. In fact, the response surface plot in Fig. 4 succinctly describes the influence of process variables on Inc of CA: only T and F_R have significant effects.

ANOVA indicated a significant ($p < 0.0007$) relationship between process variables and CA Inc but the coefficient of determination ($R^2 = 0.54$) was much lower than that of the single-bed model ($R^2 = 0.79$), suggesting that the model fit was poor. The two-bed reactor had considerably more variation in replicate measurements than the single bed design which clearly influenced the fit of the resulting model. The primary purpose of this work was to examine the effect of process variables on CA Inc, rather than create a model useful for prediction of responses under conditions not tested with this experimental design. Therefore, the significant ANOVA result is much more important, highlighting the real relationship between

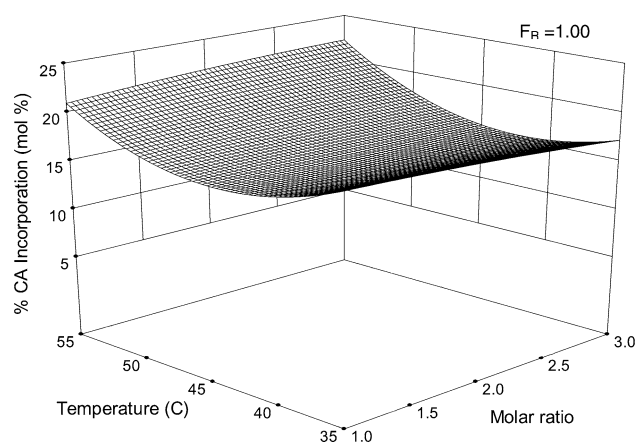


Fig. 4 Three-dimensional plot demonstrating the effects of the substrates molar ratio and reaction temperature on the predicted CA incorporation (mol %) into fish oil using a two-bed bioreactor at a constant flow rate of 1 mL/min

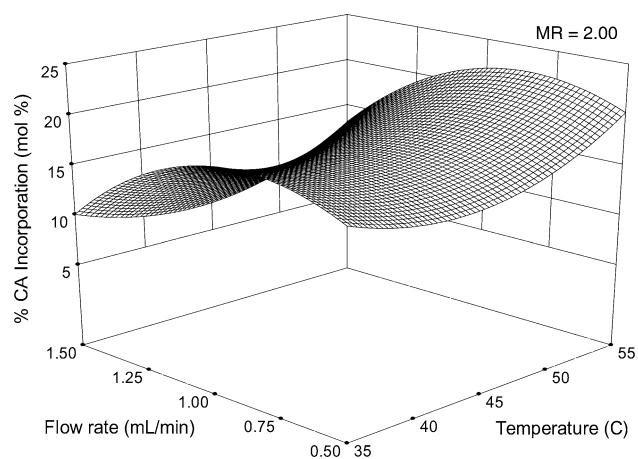


Fig. 5 Three-dimensional plot demonstrating the effects of the reaction temperature and substrates flow rate on the predicted CA incorporation (mol %) into fish oil using a two-bed reactor at a constant molar ratio of 2:1

variables and response, than the low coefficient of determination. Using this model, the maximum incorporation of CA into FO (22.7 mol%) was predicted at MR of 2.95:1 (CA/FO), at 55 °C and 0.86 mL/min.

R_T and F_R have a reciprocal relationship so that residence time can be increased or decreased by changing the volume flow rate. Here, we found that lower F_R resulted in greater Inc of CA in both reactor designs; others have similarly reported that residence time is the most critical factor in determining the extent of Inc of MCFA into SL (6, 9, 12, and 15). Intuitively, one would expect that the lowest flow rate would result in the greatest CA Inc, as was observed with the single bed design; however, with the two-bed reactor, optimal Inc of CA was predicted at 0.86 mL/min, rather than the minimum of 0.5, indicating that different mechanisms were governing extent of Inc in

the different designs. At sufficiently slow flow rates, the continuous process desired with the packed bed reactor will begin to resemble a batch process; this seems to be the effect observed with the single bed reactor. The addition of a second bed to the reactor seems to have been equivalent to increasing the reaction time of a batch process past the optimum, resulting in equilibrium being established and lower Inc of CA. If lower flow rates had been employed with the single bed reactor, it is possible that the same effect would have been observed. Unfortunately, with our reactor design, it was not possible to maintain an accurate flow rate below 0.5 mL/min and we were unable to investigate this potential effect.

In the dual-bed reactor, the relationship between CA Inc and temperature was more complex than that with the single bed, with a minimum in Inc reached with intermediate temperatures. This is in clear contrast to the straightforward positive relationship between temperature and CA Inc in the single bed design. A rationale for this unusual behavior is difficult to envision but it seems to be related to the dual-bed design.

Comparison of the Two Designs

The operation of these two different designs can be evaluated by examining process stability and productivity. The operational stability of immobilized lipases depends on many factors, including the immobilized enzyme itself, the water content of the fats, different reaction conditions and the presence of oxidation products [21, 22]. In this study, the stability of immobilized lipase from *R. mucor* was measured simply by monitoring the Inc of CA into FO under identical reaction conditions (i.e., 0.5 mL/min F_R , 35 °C, 1:1 MR) at different time intervals over the 5-week period while running the bioreactors for optimization purposes. In both reactors, the stability of this enzyme did fluctuate over the 5 week study (Table 3) but without a clear pattern. With the single bed reactor, a maximum in CA Inc was observed at the middle time point, while highest Inc was achieved at the end of the testing with the

Table 3 The stability of immobilized lipase from *R. mucor* packed in one- and two-bed reactors

PBR	%CA Inc into FO (mol%)		
	1st week	3rd week	5th week
One-bed	11.6 ± 1.2 ^{ab}	15.5 ± 0.5 ^c	12.5 ± 0.5 ^{ab}
Two-bed	18.8 ± 0.3 ^a	13.3 ± 0.7 ^b	21.0 ± 0.2 ^c

Stability was measured by monitoring the Inc of CA into FO under identical reaction conditions (i.e., 0.5 mL/min F_R , 35 °C, 1:1 MR) at different time intervals over a 5-week period (mean ± SD)

Values in each row with different roman superscript letters (a, b, c) are different ($p < 0.05$)

dual bed. Ergun's equation shows that pressure drop varies directly with length of a packed bed so that the dual bed design would have a pressure drop twice that of the single bed design. A greater pressure drop means that the packing material was experiencing much great shear forces which might lead to damage of the immobilized enzyme. However, with greatest CA Inc at the last time point in the dual-bed reactor, we know that there could not have been damage to the enzyme, eliminating pressure drop as the source of our poor reproducibility. Indeed, the packed bed columns included substantial plugs of glass wool to minimize such drops. A more likely explanation for our reproducibility issues is the difficulty encountered in maintaining a constant flow rate. The fluctuation in flow rate of reactants could also be related to external mass transfer limitations, specifically in the transport of the reactants from the substrates container to the vicinity of the enzyme. These limitations could be overcome by increasing the flow rate of substrates, but our results clearly show that lower flow rates lead to higher Inc of CA. Therefore, a compromise has to be made concerning flow rates/residence times and mass transfer limitations.

Productivity in this study can be interpreted as %Inc of CA into FO. Under optimal conditions, the one-bed model predicted higher %Inc of CA into FO than that of the two enzyme beds (31.7 vs. 22.7). These results disagree with expectation because increasing the number of beds increases the R_T and, hence, more interactions between the enzyme and the substrates should occur. Over time, the greater pressure drop in the dual bed design might be expected to lead to lower productivity due to enzyme damage but we saw no evidence of such an effect. Low CA Inc was immediately apparent at the beginning of the 45 experiments, also suggesting that other factors may have played a role. For instance, it might be that the actual reactor design used here was not optimal for a two-bed model (two columns were attached in series). The substrate mixture eluted from the first bed was fed downward into the second column and may have compressed the packed enzyme.

Alternatively, in the bioreactor with two beds, it may be that equilibrium was actually reached between CA FFA and the released FFA originally present in the TAG. In this situation, it would not be possible to increase the CA Inc by additional process optimization. Increases in MR of FFA to TAG are typically used to shift the equilibrium to favor products; when reaction time is sufficiently long and equilibrium is reached, MR will have no effect on Inc of CA [9]. This is precisely the result we obtained with the two-bed reactor. In contrast, with the single-bed reactor and the shorter residence time, MR had a significant effect on CA Inc, indicating that equilibrium was likely not reached. The shorter residence time with the single bed

design likely maintained an excess of CA and prevented the reverse reaction from occurring. Isolation and identification of FFA in the reactor eluent would indicate if CA proportions remained high.

To determine the effect of enzyme water activity (a_w) on stability and productivity, a test-tube level experiment mimicking our reactor conditions was carried out, while monitoring water activity and CA Inc over a similar 5-week period under storage conditions (immobilized enzyme stored dry at 4–5 °C). Results of this study (data not shown) showed no significant difference in CA Inc over time, despite a fluctuation in water activity from 0.196 to 0.583, and no clear relationship between enzyme stability and water activity was apparent. However, increased water activity did increase variation among replicate runs. This enzyme (*R. miehei*) is known to be more tolerant of a low water activity, thus lending further support to the findings of Yahya et al. [23] who reported that lipases from molds (e.g. *R. mucor*) seem to be more tolerant of low a_w than bacterial lipases (e.g. *Pseudomonas sp.*). Furthermore, stability may have been further enhanced here because of the ability of the support material (weak anion-exchange) to retain the appropriate level of water for the enzyme to remain active. Others have found similar results [24].

Fatty acid Profiles of Original FO and SL

The FA profile of FO before and after enzymatic modification with CA is shown in Table 4. The selected results display the FA composition of the oil under conditions approaching the optimal for maximum Inc of CA in the single-bed reactor; results for the same conditions (i.e., 0.5 mL/min F_R , 55 °C, 3:1 MR) in the dual bed design are also shown. When comparing the original FO and the resulting SL from the single-bed reactor, a significant (t test; $p < 0.001$) decrease is clear in all FA (Table 4). When results for the single-bed reactor are compared to the dual-bed design, we see additional support for the theory that equilibrium had actually been reached in the dual-bed reactor. Since the dual-bed reactor simply represents a single-bed design with an additional enzyme column, we can consider the results from the single bed to be equivalent to the midpoint of the process occurring in the two-bed reactor. All FA that decreased in amount with the one-bed reactor (14:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 18:1n-7) actually increased with the two-bed design so that their levels were more similar to that found in the original oil. In particular, proportions of 20:5n-3 were almost identical to that found in the original oil. Combined with a lower level of Inc of CA, these results suggest that many of the FA that had been cleaved from the fish oil and replaced by CA were re-esterified in the second bed of the reactor, with some loss of esterified CA. An increase in residence time with

Table 4 Fatty acid profile of lipase-assisted acidolysis of fish oil and capric acid under identical conditions (i.e., 0.5 mL/min F_R , 55 °C, 3:1 MR) in bioreactors with one or two enzyme columns (mean \pm SD)

%CA Inc into FO (mol%)			
Fatty acid	Original oil	One bed	Two beds
10:0	–	31.8 \pm 7.0 ^b	18.0 \pm 0.8 ^a
14:0	8.7 \pm 0.0 ^c	6.5 \pm 0.4 ^a	7.1 \pm 0.1 ^b
16:0	17.6 \pm 0.0 ^c	11.0 \pm 1.00 ^a	13.5 \pm 0.2 ^b
16:1n-7	9.0 \pm 0.0 ^c	5.6 \pm 0.6 ^a	6.7 \pm 0.1 ^b
16:4n-1	2.6 \pm 0.0 ^{cb}	2.1 \pm 0.1 ^a	2.4 \pm 0.0 ^{bc}
18:0	3.1 \pm 0.0 ^c	1.5 \pm 0.3 ^a	2.1 \pm 0.1 ^b
18:1n-9	8.6 \pm 0.0 ^c	3.8 \pm 0.6 ^a	5.5 \pm 0.1 ^b
18:1n-7	2.7 \pm 0.0 ^c	1.4 \pm 0.3 ^{ab}	1.8 \pm 0.1 ^{ba}
18:4n-3	2.5 \pm 0.0 ^{cb}	2.2 \pm 0.1 ^a	2.5 \pm 0.0 ^{bc}
20:5n-3	16.2 \pm 0.0 ^{cb}	11.6 \pm 1.6 ^a	14.7 \pm 0.0 ^{bc}
22:5n-3	1.6 \pm 0.0 ^{cb}	1.4 \pm 0.1 ^a	1.6 \pm 0.0 ^{bc}
22:6n-3	9.1 \pm 0.0 ^{cb}	8.3 \pm 0.2 ^a	9.0 \pm 0.1 ^{bc}

Values in each row with different roman superscript letters (a, b, c) are different ($p < 0.05$)

the second bed seems to have promoted the establishment of an equilibrium that was unfavorable for the Inc of CA.

The SL produced in this study contained a mixture of a MCFA (CA, 31.7 mol%), for rapid energy release, and long-chain n-3 PUFA (18:4n-3, 20:5 n-3, 22:5n-3, and 22:6n-3, 23.5 mol%), acting as a source of essential FA. Positional distribution of FA in a TAG molecule has a significant impact on the metabolic fate of dietary lipids, particularly when a MCFA (e.g. CA) is located at the terminal positions and PUFA are esterified to the middle position. FA at the *sn*-1 and *sn*-3 positions are hydrolyzed by pancreatic lipase and quickly absorbed and oxidized/metabolized for energy while those at the *sn*-2 position remain unchanged and are used in the synthesis of new TAG. Thus, the SL created here may have use in applications where quick energy release and n-3 supplementation are required. However, the necessary *in vivo* studies, demonstrating enhanced intake of n-3 FA and beneficial effects on blood lipid profile from such SL have yet to be carried out.

Conclusions

PBR with one or two enzyme beds were constructed for the production of specialty lipids via lipase-assisted acidolysis of FO and CA. Immobilized lipase from *R. miehei* was used as a biocatalyst in a solvent-free environment. RSM was applied to the models and used to optimize the selected reaction conditions. In general, under the conditions of low substrate molar ratio (1:1–3:1 CA/FO), the one-bed model was significantly affected by all reaction conditions. In the

two-enzyme bed model, T and F_R had significant effects on Inc of CA; MR had no effect, suggesting that the longer residence time had permitted equilibrium to be attained. The maximum Inc of CA into FO (31.7 mol%) using the single enzyme column was predicted at MR, T and F_R of 2.70:1 (CA/FO), 55 °C and 0.5 mL/min, respectively. The corresponding maximum Inc of CA into FO (22.7 mol%) using the dual column design was predicted at substrates MR of 2.95:1 (CA/FO), at 55 °C and 0.86 mL/min, under the conditions of low substrate molar ratio (1:1–3:1 CA/FO). Assessing the effect of process factors on CA Inc into FO was the main objective of this study, rather than generating a model useful for prediction of responses under conditions not examined with this design. Thus, the significant ANOVA results are much more valuable, underlining the actual relationship between parameters and response, despite the low R^2 . The bioreactor with one bed was more efficient than the two-column design when considering the reaction yield, amount of enzyme, shorter residence time, lower amount of substrates, and the overall economy of the process. The system described here did not require any organic solvents for the reaction, resulting in reduced overall cost and a 'green' process. The SL produced contained a unique combination of a MCFA (CA) esterified to the terminal positions, for rapid energy release, and retained considerable amounts of long-chain PUFA, acting as a source of essential FA.

Acknowledgments We are grateful to the Natural Sciences and Engineering Research Council (NSERC) of Canada for financial support. Samples of fish oil were kindly provided by Ascenta Health (Dartmouth, NS, Canada).

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