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Synthesis of Structured Triacylglycerols by Lipase-Catalyzed Acidolysis in a Packed Bed Bioreactor

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Structured triacylglycerols (ST) from canola oil were produced by enzymatic acidolysis in a packed bed bioreactor. A commercially immobilized 1,3-specific lipase, Lipozyme IM, from *Rhizomucor miehei*, was the biocatalyst and caprylic acid the acyl donor. Parameters such as substrate flow rate, substrate molar ratio, reaction temperature, and substrate water content were examined. High-performance liquid chromatography was used to monitor the reaction and product yields. The study showed that all of the parameters had effects on the yields of the expected di-incorporated (dicaprylic) ST products. Flow rates below 1 mL/min led to reaction equilibrium, and lower flow rates did not raise the incorporation of caprylic acid and the product yield. Incorporation of caprylic acid and the targeted di-incorporated ST was increased by ~20% with temperature increase from 40 to 70 °C. Increasing the substrate molar ratio from 1:1 to 7:1 increased the incorporation of caprylic acid and the product yield slightly. Water content in the substrate also had a mild influence on the reaction. Water content at 0.08% added to the substrate gave the lowest incorporation and product yield. The use of solvent in the medium was also studied, and results demonstrated that it did not increase the reaction rate at 55 °C when 33% hexane (v/v) was added. The main fatty acids at the sn-2 position of the ST were $C_{18:1}$, 54.7 mol %; $C_{18:2}$, 30.7 mol %; and $C_{18:3}$, 11.0 mol %.

Keywords: *HPLC; lipase-catalyzed acidolysis; Lipozyme IM; packed bed reactor; Rhizomucor miehei; structured triacylglycerols*

INTRODUCTION

The applications of lipases in oil and fat modifications are promising in many aspects. The most important and obvious merits are (1) the efficacy of lipases under mild reaction conditions, (2) the catalysis of specific reactions, (3) their utility in "natural" reaction systems and products, (4) reduced environmental pollution, (5) the availability of lipases from a wide range of sources, (6) the ability to improve lipases by genetic engineering, and, in special situations, (7) the use of lipases for the production of particular biomolecules. For these reasons, many nutritional and functional lipids have been produced enzymatically, and a great many of these studies have been published in the past 20 years. A few reviews have been published recently (Mukherjee, 1990; Eigtved, 1992; Akoh, 1996; Ramamurthi and McCurdy, 1996). Structured triacylglycerols (ST), in this paper, refer to products containing both long-chain oleic and essential fatty acids such as linoleic and linolenic acids, which are located specifically at the sn-2 position, and medium-chain fatty acids such as caprylic acids, which are located mainly at the sn-1,3 positions of the glycerol backbone. ST of such kind are not available in nature and cannot be produced by chemical methods. For commercial development, they can be produced only by enzymatic methods using sn-1,3 specific lipases. The nutritional applications of ST have recently attracted attention (Xu et al., 1997; Jandacek et al., 1987; Ikeda et al., 1991; Christensen et al., 1995a,b; Jensen et al., 1995), resulting in increased interest in the production of these fats by lipase-catalyzed reactions (Macrae, 1992; Quinlan and Moore, 1993; Akoh, 1995).

Packed bed bioreactors have been investigated and applied in the lipase-catalyzed lipid modifications, and a few papers have been published on the applications of fixed bed bioreactors (Forssel et al., 1993; Luck and Bauer, 1991; Posorske et al., 1988; Hansen and Eigtved, 1987; Jung and Bauer, 1992, Mu et al., 1998; Xu et al., 1998). Hansen and Eigtved (1987) and Posorske et al. (1988) investigated solvent-free fat modification in a packed bed reactor using Lipozyme. The immobilized lipase was stable for up to 3 months, and tons of products can be produced with 1 kg of the biocatalyst. Forssel et al. (1993) also investigated some of the features of enzymatic transesterification of rapeseed oil and lauric acid in a continuous reactor. Recently, Mu et al. (1998) and Xu et al. (1998) investigated the production of specific-structured lipids in enzyme bed

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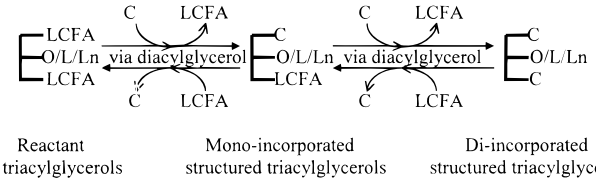

Figure 1. Reaction scheme for the Lipozyme IM-catalyzed acidolysis between canola oil and caprylic acid. LCFA, long-chain fatty acids from canola oil; C, caprylic acid; O, oleic acid; L, linoleic acid; Ln, linolenic acid.

reactors and were able to scale up to kilogram production in their pilot plant. All of the above work demonstrates that fixed bed bioreactors are very promising for future developments for lipase-catalyzed lipid modifications with or without solvents.

In this paper, ST from canola oil were synthesized in a packed bed bioreactor. The reaction mechanism is depicted in Figure 1. The reaction and product yield were monitored by high-performance liquid chromatography (HPLC). Parameters such as flow rate, reaction temperature, substrate molar ratio, and water content were investigated, and some of the other possible influences were also examined. The ST were produced under optimal conditions, and the product mixture was purified by short-path distillation. The characteristics of the packed bed bioreactor were also compared to those used in previous publications.

MATERIALS AND METHODS

Materials. Refined, bleached, and deodorized (RBD) canola oil was purchased from the local supermarket. The fatty acid composition (mol %) of the canola oil was as follows: $C_{16:0}$, 5.0; $C_{18:0}$, 2.5; $C_{18:1n-9}$, 61.9; $C_{18:2n-6}$, 22.0; $C_{18:3n-3}$, 7.6; others, 1.0. The free fatty acid content of the canola oil was 0.02%, and the peroxide value was 0.0 mequiv/kg (undetected). The water content was 0.03%. Caprylic acid was purchased from Sigma Chemical Co. (St. Louis, MO) (purity minimum 99% and water content 0.05%). Lipozyme IM, a commercially immobilized 1,3-specific lipase from *Rhizomucor miehei*, was from Novo Nordisk A/S, Bagsvaerd, Denmark (water content = 2−3 wt %). All solvents and reagents for analyses were chromatographic or analytical grade.

Apparatus. The setup of the bioreactor was similar to that previously reported (Xu et al., 1998). The bed of the reactor was, however, made of stainless steel with the dimensions of 47 mm (i.d.) × 50 cm. An FMI Lab pump (model QV) from Fluid Metering, Inc. (New York, NY), was used to feed the substrate. The bed was jacketed, and the temperature was maintained by a circulating water bath. Before the substrates were pumped into the enzyme bed, they were preheated to a set temperature by a coiled heater. The bioreactor contained temperature and pressure probes at both the inlet and outlet of the bioreactor, which were connected to a computer, for monitoring purposes.

Packing of the Column. The bed was packed with the immobilized lipase by loading the prepared enzyme particles directly into the column. The upper and lower ends of the bed were layered each with glass wool (3 and 7 cm in thickness, respectively). Nitrogen was passed through the packed bed to remove air. The substrates were then pumped through to condition the bed for a few hours before parameter study.

Experimental Methods. Experiments on lipase-catalyzed acidolysis were performed with canola oil and caprylic acid (1-kg scale). When a new experiment was started, prepared substrates were pumped into the enzyme bed at the set parameters. Samples were taken after 800-mL products were collected under the same set parameters. The final production (2 kg) was carried out under optimal conditions.

Product Purification by Short-Path Distillation. The product mixture was purified by laboratory short-path distillation equipment (UIC Inc., Joliet, IL). The purification was done in two stages by separating the caprylic acid for the first stage and the long-chain fatty acids for the second stage. The conditions for the first stage are as follows: heating oil temperature, 75 °C; cooling water temperature, 15 °C; mixture holding temperature, 40 °C. The conditions for the second stage are as follows: heating oil temperature, 185 °C; cooling water temperature, 20 °C; mixture holding temperature, 40 °C. In both cases, the pressure was controlled below 2×10^{-2} Torr.

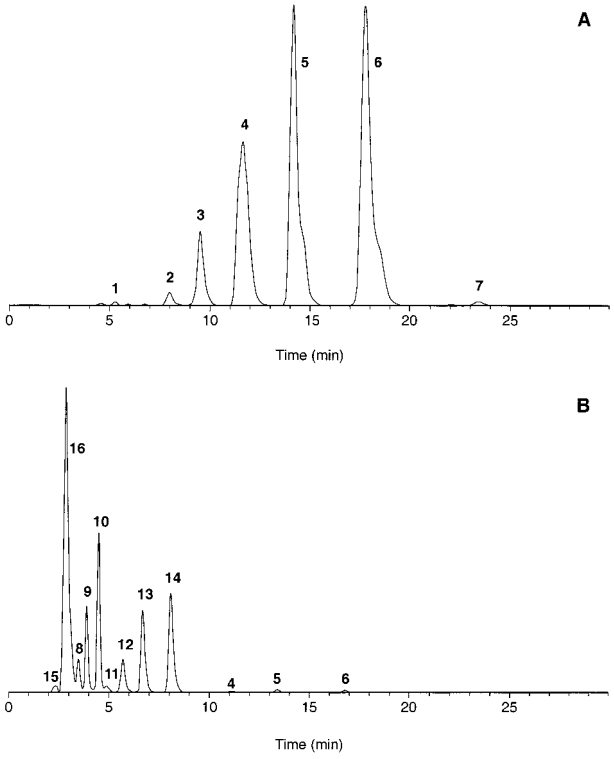

Figure 2. HPLC separation chromatograms for unmodified canola oil (A) and the structured triacylglycerols product mixture from the packed bed bioreactor (B) under the chromatographic conditions described under Materials and Methods. Peaks 1−14 are identified in Tables 1 and 2. Peaks 15 and 16 were identified as caprylic acid and long-chain fatty acids from canola oil with standards.

Triacylglycerol Composition Analysis by HPLC. Samples (100 mg each) were dissolved in 3 mL of acetone. Then, 1 mL of the solution was transferred into the vial for analysis. HPLC was performed with a Hewlett-Packard (Avondale, PA) model 1090 Win liquid chromatograph equipped with a Sedex 45 evaporative light scattering detector (ELSD; Richard Scientific, Novato, CA) and a Vectra computer. The ELSD was set to 40 °C at a nitrogen nebulizer gas pressure of 2.1 and a gain of 5 for the reverse phase system. A reverse phase HPLC column, Ultrasphere ODS 5 μm from Beckman/Altex (San Ramon, CA), was used with the dimension of 4.6 mm × 25 cm. The column temperature was set to 45 °C. A mixture of acetonitrile and acetone (36.4:63.6, v/v) was used as the liquid phase with an initial flow rate of 1.2 mL/min, increased to 1.5 mL/min after 5 min, and maintained at this flow rate for the remainder of the run. Total run time was 25 min. The separation of canola oil and the product mixture from the bioreactor can be seen in Figure 2. The concentration of each relevant compound was recalculated into molar percentages on the basis of the area percentages as described before (Xu et al., 1999a). The incorporation (Inc) of caprylic acid into the sn-1 and -3 positions of canola oil was calculated by the equation (Bloomer et al., 1991)

$$\text{Inc (mol \%)} = \{[Y] + 2[Z]/2([X] + [Y] + [Z])\} \times 100\%$$

where [X] is molar content of nonincorporated triacylglycerols (NST) from canola oil, [Y] is monoincorporated triacylglycerols

Table 1. Calculated Triacylglycerol Species in Canola Oil and Their Contents Classified by Their ECN and Corresponding Peaks in Figure 2A[a]

peak (HPLC):	1	2	3	4	5	6	7
ECN:	38	40	42	44	46	48	50
TAGs and contents:	LLnLn 0.22 LnLLn 0.11	OLnLn 0.84 LLLn 0.62 LLnL 0.31 LnOLn 0.18 PLnLn 0.10	OLnL 2.44 LOLn 1.04 OLLn 2.44 LLL 0.90 PLnL 0.28 PLLn 0.28 LnLLn 0.11	OLL 7.08 OLnO 4.82 OOLn 4.12 OLnP 1.12 LOL 1.51 PLL 0.82 POLn 0.48 PLnP 0.12	OLO 13.96 OOL 11.94 OLP 3.22 LOP 1.38 OLnS 0.56 SLL 0.40 PLP 0.19	OOO 23.53 OOP 5.42 OLS 1.60 SOL 0.68 POP 0.31 SLP 0.18	SOO 2.72 POS 0.32
calcd:	0.3	2.0	8.4	20.1	31.6	31.7	3.0
anal.:	0.1	1.0	5.8	21.4	31.6	39.6	0.5

[a] Abbreviations: ECN, equivalent carbon number; TAG, triacylglycerol; P, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; Ln, linolenic acid; calcd, calculated; anal., analyzed. Only the 2-position is specified in all of the written triacylglycerols (β-form).

(MST), and [Z] is di-incorporated triacylglycerols (DST). Duplicate measurements were made, and the average was taken for all of the analysis results.

TLC Detection and Determination of Diacylglycerol Content. A general procedure similar to the one previously described was used in this case (Lee and Akoh, 1996). A 20 × 20 cm plate coated with silica gel G (Fisher Scientific) was used for the separation. The developing solvent used was petroleum ether/ethyl ether/acetone (90:10:2, v/v/v). A 0.2% methanol solution of 2,7-dichlorofluorescein was used to visualize the band.

Fatty Acid Composition Analysis. The fatty acid compositions of canola oil and the purified product were analyzed by gas chromatography (GC) as previously described (Fomuso and Akoh, 1996). Fatty acid methyl esters were prepared by methylation. The fatty acid methyl esters were extracted with hexane and analyzed by GC.

Grignard Analysis. The fatty acid distribution at the sn-2 position of the final product was determined by Grignard degradation with allylmagnesium bromide followed by isolation, methylation, and fatty acid composition analysis of the sn-2 monoacylglycerol fraction (Becker et al., 1993).

Other Analyses. The water content of canola oil and caprylic acid was determined by using the Karl Fischer method (720 KFS Titrino, Switzerland, using Hydranal titrant and solvents). The free fatty acid content and peroxide value were determined by using the alkali titration method and the thiosulfate titration method, respectively (AOAC, 1990).

RESULTS AND DISCUSSION

HPLC Separation and Peak Identification. Canola oil mainly contains oleic, linoleic, and linolenic acids, which account for >90% of the composition. The triacylglycerols of canola oil were successfully separated according to their equivalent carbon numbers (ECN) (Figure 2A). ECN was calculated as CN − 2(DN), where CN is carbon number without glycerol and DN is the number of double bonds per triacylglycerol molecule. The identification of each peak was assisted by the calculation of triacylglycerol composition. The sn-2 position fatty acid composition of the unmodified canola oil was first calculated by a previous R-distribution hypothesis (Xu, 1990) as follows (mol %): palmitic (P), 0.0; stearic (S), 0.0; oleic (O), 55.6; linoleic (L), 33.0; linolenic (Ln), 11.4; and others 0.0. The results agree well with the analyzed results from previous publications for canola oil (Gunstone et al., 1994). The distribution in sn-1,3 positions was thus calculated as follows (mol %): P (7.5), S (3.8), O (65.0), L (16.5), Ln (5.7), and others (1.5) by using the equation

$$\text{Sn-1,3 (\%)} = (3[\text{T}] - \text{sn-2})/2$$

Table 2. Peak Identification and Possible Triacylglycerol Species for MST and DST in the Canola Oil ST According to Table 1[a]

peak:	8	9	10	11	12	13	14
ECN:	28	30 (DST)	32[b]	34	36	38 (MST)	40
TAGs:	CLnC	CLC	COL	CLLn CLnL	CLnO COLn CLnP CLL	COL CLO CLP CLnS	COO COP CLS

[a] Abbreviations: DST, di-incorporated structured triacylglycerols; MST, monoincorporated structured triacylglycerols; C, caprylic acid; ST, structured triacylglycerols; for others, see Table 1. [b] The MST CLnLn can also be in this peak with the same ECN, but very low in content according to the calculated content of the possible triacylglycerols in Table 1, and therefore can be omitted.

where [T] is the fatty acid content in canola oil. The calculation of the triacylglycerol composition was based on the 1,3-random and 2-random hypotheses from Van der Waal (Bockisch, 1998). The basic equations for this calculation are

$$\beta\text{-XYZ (\%)} = \{2(\text{X in sn-1 position}) \times (\text{Y in sn-2 position}) \times (\text{Z in sn-3 position})\}/10000$$

$$\beta\text{-XYX (\%)} = \{(\text{X in sn-1 position}) \times (\text{Y in sn-2 position}) \times (\text{X in sn-3 position})\}/10000$$

$$\text{XXX (\%)} = \{(\text{X in sn-1 position}) \times (\text{X in sn-2 position}) \times (\text{X in sn-3 position})\}/10000$$

where X, Y, and Z are different fatty acids. Because the distributions at the sn-1 and sn-3 positions were treated equally and randomly, the isomers such as sn-XYZ and sn-ZYX were combined into the same species as β-XYZ. The main triacylglycerol components with >0.1% were sorted and classified by their ECN numbers, and the list is included in Table 1. As can be seen, the ECN numbers from 38 to 50 agreed with the peaks in Figure 2A, and the contents between the calculated and the HPLC results generally agree with each other. This identification is also in agreement with that in a previous publication (Salivaras and McCurdy, 1992). The triacylglycerol component calculation and identification in Table 1 will help identify the peaks in Figure 2B. From Table 1 and the reaction mechanism in Figure 1, it is easy to list the most abundant MST and DST. These incorporated species are identified in Table 2. Peaks 1 and 2 from the original canola oil may overlap with the product components (peaks 11 and 14, respectively) as shown in Figure 2B. However, because peaks

4, 5, and 6 were almost fully converted to structured lipids containing caprylic acid and peak 3 was not detected in Figure 2B, it is reasonable to assume that most of peaks 1 and 2 were converted to structured lipids. Other possible compounds, if present, that might influence the quantitation of structured lipids in peaks 8−14 are diacylglycerols. Diacylglycerols are normally formed as byproducts during the lipase-catalyzed acidolysis (Xu et al., 1999), but the amount was very small in packed bed reactors (Mu et al., 1998; Xu et al., 1998). Diacylglycerols from ECN 20 to ECN 34 may possibly exist in the present reaction; however, diacylglycerols were not detected after TLC separation and determination because of the low content. Therefore, they did not significantly influence the results of DST and MST (peaks 8−14). To confirm this identification, we calculated the average ratio for all of the samples collected for this study between peaks 8, 9, and 10, that is, the ratio of CLnC/CLC/COC, and the result is 1:2.6:4.8. This ratio agrees well with that of Ln, L, and O in the sn-2 position of the canola oil (1:2.9:4.9). This demonstrates that the identification is correct and the quantitation is satisfactory.

Packed Bed Property. As studied previously (Jung and Bauer, 1992; Xu et al., 1998), packed bed reactors were more influenced by mass transfer limitations than stirred tank reactors. This is, of course, reflected by the designs and dimensions of packed bed reactors. A comparison was made to highlight the difference between the present reactor and those reported before. As can be seen from Table 3, the ratio between the diameter of the bed and its length is larger than those previously reported. This will therefore increase the lower limit of the flow rate to reach the minimum linear flow rate. It was reported (Forssel et al., 1993) that the external mass transfer limitation could be omitted if the linear flow rate was $>3 \times 10^{-5}$ m/s (0.18 cm/min). Therefore, it is necessary to meet this lower limit to reduce the influence of the external mass transfer limitation. The relationship between the volume flow rate and the linear flow rate of the present bed is included in Figure 3. The reaction time is directly proportional to the residence time of the substrate in the bed. Residence time may not be the same as the reaction time, and it is totally dependent on the liquid flow manner and the bed behavior. Residence time is often used to represent the reaction because it can be calculated from flow rates. The residence time has a reciprocal relationship with volume flow rate, and the equation is

$$\text{residence time} = \text{bed volume} \times \text{void fraction/volume flow rate}$$

The void fraction of Lipozyme IM was reported to be 0.45 (Xu et al., 1998). Therefore, the residence times corresponding to different volume flow rates were calculated, and the relationship between residence time and volume flow rate was built into the range used in this study as given in Figure 3.

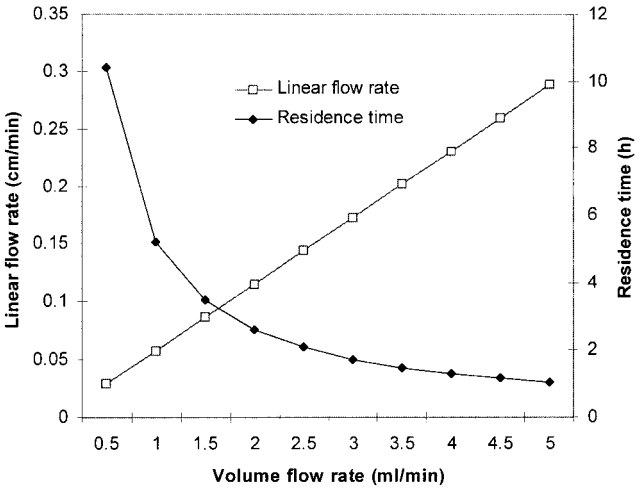

Figure 3. Relationships between volume flow rate and residence time and linear flow rate for the packed bed bioreactor as described under Materials and Methods.

Volume Flow Rate. Residence time was reported to be a most crucial parameter that was responsible for the higher yields in the packed bed bioreactor (Mu et al., 1998). Residence time directly relates to the flow rate when the packed bed is the same, as described in Figure 3. As a consequence, flow rate is one of the important parameters for the packed bed bioreactor. As reported previously (Mu et al., 1998; Xu et al., 1998), the incorporation had a normal sigmoidal curve with a residence time the same as that of Michealis−Menten kinetics between reaction rate and substrate concentration. Because residence time has a reciprocal relationship with flow rate, the curve between incorporation and flow rate was different (Figure 4). The Inc and the content of DST both had a turn at ∼1 mL/min. Below this flow rate, the reaction reached its equilibrium. The most important outcome was a decrease in DST with increased flow rate. This flow rate at the turn is equivalent to the residence time of ∼5 h according to Figure 3. The flow rate between 2 and 4 mL/min had a slight effect on Inc. This is because it results in a small change of the correspondent residence time within that range, as described in Figure 3.

Reaction Temperature. Temperature for the present reaction system has two meanings. Higher temperature will increase the reaction rate according to the general rule of the Arrhenius equation in an exponential function as

Table 3. Comparison of Packed Enzyme Bed Bioreactors for Lipase-Catalyzed Lipid Modifications

bed vol (mL)	bed length (cm)	bed diameter (cm)	ratio[a]	flow direction	other bed information	reference
417	85	2.5	0.029	downward	jacketed glass column	Posorske et al., 1988
78	25	2.0	0.080	upward	glass column	Forssel et al., 1993
123	25	2.5	0.10	upward	jacketed glass column	Sarney et al., 1994
2870	65	7.5	0.12	downward	jacketed column	Wisdom et al., 1987
24−55	30−70	1.0	0.033−0.014	downward	jacketed glass column	Luck and Bauer, 1991
202	38	2.6	0.068	upward	jacketed glass column	Mu et al., 1998
1688	86	5.0	0.058	upward	jacketed glass column	Xu et al., 1998
694	40	4.7	0.12	upward	jacketed stainless steel column	present study

[a] The ratio between bed diameter and bed length.

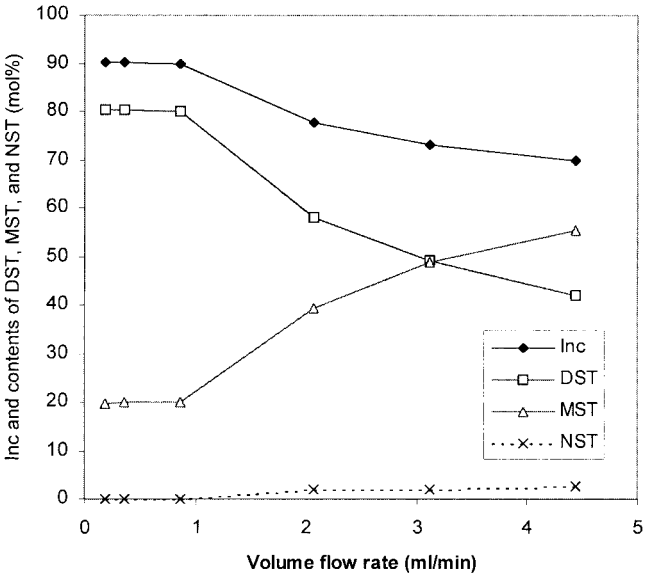

Figure 4. Effect of flow rate on the incorporation of caprylic acid (Inc) and contents of di-incorporated structured triacylglycerols (DST), monoincorporated structured triacylglycerols (MST), and nonincorporated triacylglycerols (NST). Other conditions were as follows: temperature, 55 °C; substrate molar ratio, 6:1; water content, 0.04% added.

$$k = A \exp(-E/RT)$$

where A is a constant, E is activation energy, R is the gas constant, k is the rate, and T is temperature. Temperature is also related to the mass transfer limitations in the packed bed. Higher temperature reduces the viscosity of the lipid mixture and certainly increases the substrate and product transfer on the surface or inside the enzyme particles. In this study (Figure 5), the incorporation of caprylic acid (Inc) and the content of DST were increased by ~20% with a temperature increase from 40 to 70 °C. The content of MST showed slight change. The reaction was not influenced much by temperature in the range between 50 and 60 °C compared to other temperature ranges. As reported before (Boy et al., 1998), lipase stability was also influenced by temperature. A higher temperature will greatly reduce the enzyme stability and its half-life. Furthermore, higher temperature will increase the lipid oxidation rate, especially for polyunsaturated fatty acids. Therefore, temperatures between 50 and 60 °C are recommended for canola oil and other polyunsaturated oils that have low melting points.

Substrate Molar Ratio. Substrate molar ratio also had double functions (Xu et al., 1998). Higher substrate ratios will raise the reaction equilibrium and increase the theoretical maximum incorporation and product yields. On the other hand, higher free fatty acid content will increase the inhibition effect and lead to longer reaction time to reach equilibrium. In this study (Figure 6), Inc and the content of DST were increased by the increase of substrate molar ratio, but the change was very small. The reaction was also related to other parameters as mentioned above, especially residence time or flow rate. The conclusion can be different if other parameters are used.

Water Content. A small amount of water is essential in the lipase-catalyzed acidolysis, to maintain the lipase structure and activate the lipase function, and is involved in the initial reaction according to the sequential hydrolysis and esterification mechanism (Reyes and

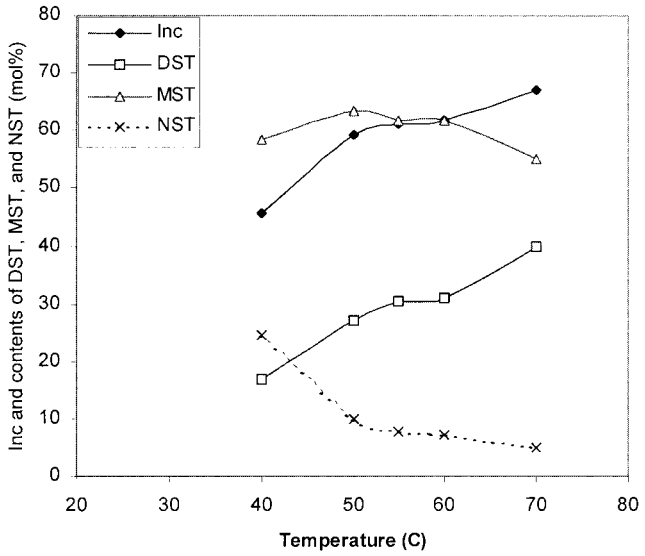

Figure 5. Effect of temperature on Inc and contents of DST, MST, and NST. Other conditions were as follows: flow rate, 4.05 mL/min; substrate molar ratio, 3:1; water content, 0.04% added. For abbreviations see Figure 4.

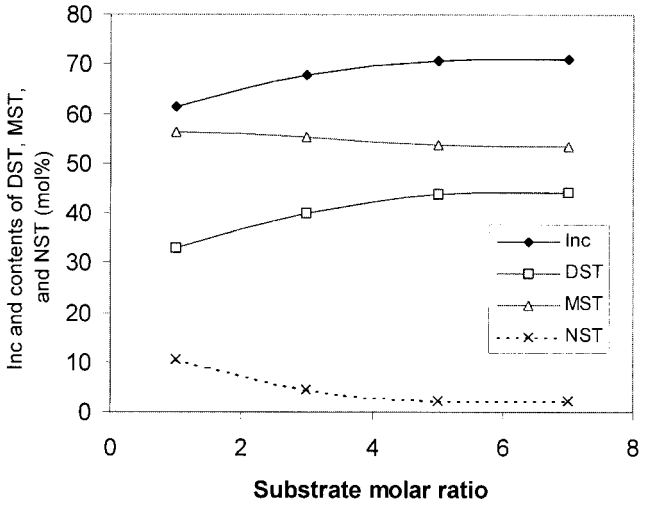

Figure 6. Effect of substrate molar ratio on Inc and contents of DST, MST, and NST. Other conditions were as follows: flow rate, 3.20 mL/min; temperature, 55 °C; water content, 0.04% added. For abbreviations see Figure 4.

Hill, 1994). On the other hand, it was reported (Mu et al., 1998) that water content variation in the substrate had little effect on the incorporation and product yields. This phenomenon was also proved in a pilot scale study (Xu et al., 1999a). Water in the packed bed might not directly function as supplied in the substrate stream. Water could be stored in the enzyme particles due to enzyme binding and carrier absorption and then supplied later when the stream had less water content. This effect may reduce the difference between water contents in the substrate. It was assumed that a longer reaction interval might depress this "buffer" effect of water. However, enzyme stability will have a large effect on the results at longer intervals. In this study (Figure 7), the running time was relatively prolonged. The results showed that water content had some effects on Inc and the content of DST, but the effects were comparably small. The added water content at 0.08% gave the lowest Inc and content of DST. The reason for this result needs further study.

Ratio between DST and MST. The ratio between

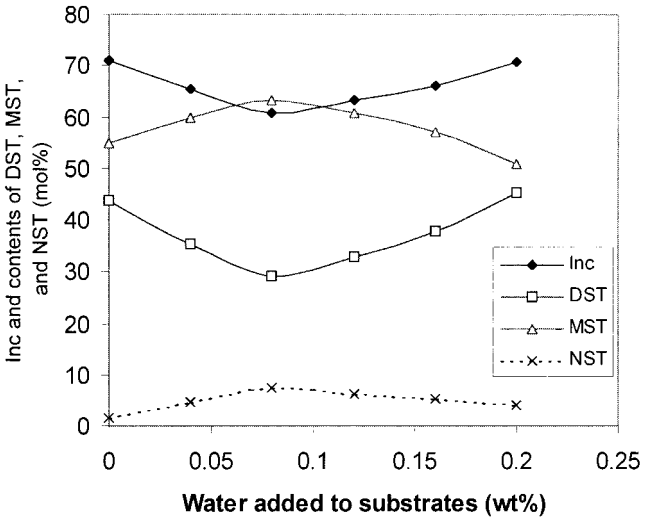

Figure 7. Effect of water content in the substrates on Inc and the contents of DST, MST, and NST. Other conditions were as follows: flow rate, 2.82 mL/min; temperature, 55 °C; substrate molar ratioo, 3:1. For abbreviations see Figure 4.

the contents of DST and MST has a few implications for understanding the reaction. The reaction mechanism in Figure 1 is a step-by-step process, and the reaction is reversible. When the same substrate ratio is used and the equilibrium is reached, the ratios between the contents of DST and MST should be the same. If the reaction mechanism is not a step-by-step process and the formation of DST can occur directly from canola oil, the ratios will be different. Mu et al. (1998) found that the content of DST still increased when the residence time was prolonged, even though the incorporation of acyl donors remained constant. This demonstrates that the reaction may tend to form DST instead of MST. In the present study, the ratio decreased dramatically by the increase of flow rate and increased slightly by temperature and substrate molar ratio increase (Figure 8). The effect of water content on the ratio had a tendency similar to that in Figure 4. From Figure 8, it can be seen that flow rate had the greatest influence on the reaction as observed before for residence time (Mu et al., 1998).

Use of Hexane as the Reaction Medium. The use of solvent is mainly to reduce the viscosity of lipids in this specific system. The other merits of using a solvent system were reported to be the easy control of humidity of the reaction system and the possible extraction of produced or released components from the reaction system to force the reaction toward the end (Brink et al., 1988). It was also reported that enzyme, if optimally applied, works well in solvent systems as in aqueous systems (Klibanov, 1997). We studied the effect of flow rate on reactions involving hexane (substrate/hexane = 2:1 by volume). The hexane was saturated with water before blending with the substrate. The same conditions were used as in Figure 4. It can be seen that the reaction was similar to that in Figure 4, but the reaction rate was slower (Figure 9). This was probably caused by the diluted or lower concentration of the substrate. Therefore, we conclude that the use of solvent has no special function under relatively high temperature or the viscosity of substrate is low enough under the temperature used to preclude the use of solvent.

Other Process Features. The products were synthesized under different stages. The product from the first stage (1.31 mL/min) was re-passed through the packed bed reactor under different flow rates (3.28 and 2.80 mL/min). Other conditions were the same: temperature, 55 °C; substrate molar ratio, 6:1; water content, 0.10% added. The first pass resulted in Inc of 83.1% and the content of DST of 66.2%. The second pass under a faster flow rate resulted in reduced Inc and content of DST (77.7 and 55.3%, respectively). The second pass did not increase the yield; on the contrary, it decreased the yield. The second pass with a slower flow rate increased the incorporation (Inc 89.6%) and the yield of the expected ST (DST 79.1%), but the increases were very small. We conclude from this result that the yields in a two-stage reaction are not simply the direct addition of two yields from individual passes even though the total residence time is the same.

The cleaning of the enzyme bed by hexane was also investigated to check its influence. Reaction conditions were the same before and after the cleaning: flow rate, 1.90 mL/min; temperature, 55 °C; substrate molar ratio, 3:1; water content, 0.10% added. The results for Inc and the contents of DST, MST, and NST before the cleaning were 73.4, 49.7, 47.5, and 2.9%, respectively, and after the cleaning were 74.3, 50.1, 48.6, and 1.4%, respectively. The activity of the enzyme in the bed slightly increased after cleaning of the bed with hexane. It is

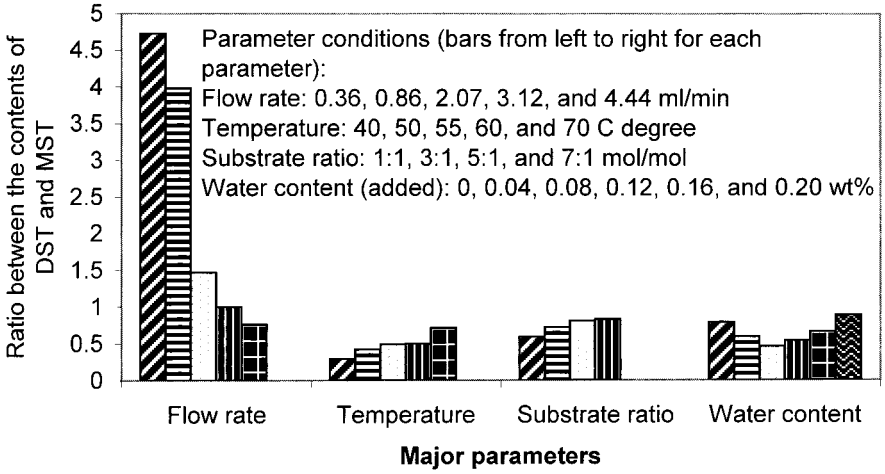

Figure 8. Effects of the four major parameters on the ratio between the contents of DST and MST. Other conditions were the same as those described in Figures 4, 5, 6, and 7, respectively. For abbreviations see Figure 4.

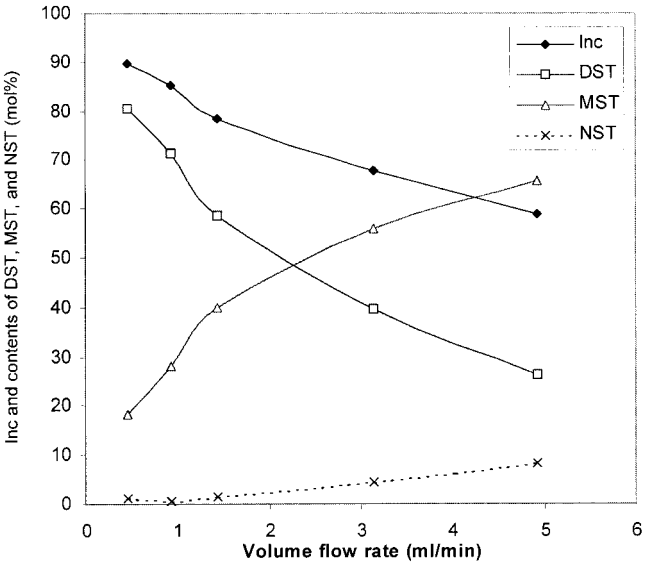

Figure 9. Effect of flow rate on Inc and contents of DST, MST, and NST when reaction was performed in hexane. Substrates were homogeneously blended with water-saturated hexane (2:1, v/v). Other conditions were as follows: temperature, 55 °C; substrate molar ratio, 6:1; water content, 0.04% added to the substrate before blending with hexane.

therefore possible and useful to clean the packed enzyme bed between different productions without loss of activity.

Production of ST under Optimal Conditions. The final production (2 kg) was performed under optimal conditions from the above parameter studies. The optimal conditions were selected as follows: flow rate, 1 mL/min; temperature, 60 °C; substrate molar ratio, 5:1; water content, 0.20% added. The product was purified by short-path distillation, and the final product was characterized. The fatty acid composition of the final product was as follows (mol %): C, 40.1; P, 2.0; S, 1.3; O, 34.7; L, 15.8; Ln, 5.4; others, 0.7. The fatty acid distribution in sn-2 position was as follows (mol %): C, 3.4; P, 0.2; O, 54.7; L, 30.7; Ln, 11.0. The other characteristics are free fatty acid content of 0.18 wt % and peroxide value of 0.0 mequiv/kg. As can be seen from these results, the production of 2 kg of the ST agrees well with the parameter study and the product has a high quality.

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