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Production of Glycerides from Glycerol and Fatty Acids by Native Lipase of *Nigella sativa* Seed

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ABSTRACT

The possible application of native lipase of *Nigella sativa* seed in the esterification of fatty acids to glycerol was investigated, and the effect of process parameters and the enzyme selectivity on the reaction were determined. For this aim, the esterification of oleic acid, sunflower oil fatty acids, and coco oil fatty acids with glycerol were studied.

Index Entries: *Nigella sativa* seed; lipase; esterification, lipase selectivity; sunflower oil fatty acids; coco oil fatty acids.

INTRODUCTION

The esterification of fatty acids to glycerol was known as early as 1844 (*1*). This reaction is carried out either catalytically or in the absence of a catalyst at 453–503 K, while under subatmospheric pressure or while being stripped with an inert gas (*1*). These processes are energy-intensive and give rise to a variety of undesirable side reactions. The removal of the catalysts and undesirable products from the ester products is a major problem that at best involves yield losses owing to subsequent bleaching or refining operations. Enzyme-catalyzed esterification reactions are superior to these conventional chemical esterification methods owing to mild reaction conditions, high catalytic efficiency, and selectivity of

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catalyst, which produces purer products. The use of lipolytic enzymes to catalyze the esterification reaction is being actively investigated by many workers (2–5). Lipases are present at high activity in many oil seeds (6). Some of the lipases have catalytic activity only on the hydrolysis reaction of fats and oils, and some of them have activity on both the hydrolysis and esterification reactions. *Nigella sativa* seed is a lipase source whose native lipase is a very active catalyst for the hydrolysis of oils (7–9).

In order to develop possible applications of the native lipase of *N. sativa* seed in esterification reactions, we have studied the enzyme-catalyzed esterification of fatty acids to glycerol and investigated the effect of process parameters, such as temperature, concentration of lipase, reactant ratio, and fatty acid composition, on the reaction.

MATERIALS AND METHODS

Material

N. sativa seeds of Turkish origin were purchased locally. The oil content of seeds on moisture-free basis were 36.3%; moisture, 7.42%. Oleic acid and glycerol (87%) were obtained from Merck Co. (Darmstadt, Germany). Sunflower oil and coco oil fatty acids were purchased from Alemdar Chemical Co. (Istanbul, Turkey). These fatty acids were used directly without further purification. The main characteristics and the composition of sunflower oil and coco oil fatty acids are shown in Table 1. All the other chemicals used for experiments were analytical-grade (Merck, Darmstadt, Germany).

Apparatus

Enzymatic esterification reactions were carried out in a three-necked flask (250 mL) equipped with a sampling pipet, a temperature controller, and a vacuum pump connection.

Enzymatic Esterification of Glycerol with Fatty Acids by Native Lipase of *Nigella sativa* Seed

In all experiments, ground *N. sativa* seeds (600–1400 μm) were pressed with a laboratory-type Carver hydraulic press (Fred S. Carver Inc., Wabash, IN) at ambient temperature under the following pressure program: 69×10^6 Nm^{-2} (20 min), 103.5×10^6 Nm^{-2} (15 min), 138×10^6 Nm^{-2} (20 min). The pressed seeds were then used as catalyst. Using pressed *N. sativa* seeds as an esterification catalyst allows the seed oil to be used in the other technological applications. A certain quantity of glycerol (87%) and fatty acids was placed into the reaction flask and heated by stirring to the reaction temperature. The pressed seeds were added to the flask as a catalyst after attainment of the desired temperature, and the vacuum was

Table 1
The Main Characteristics of Fatty Acids

Species	Oleic acid	Sunflower oil fatty acids	Coco oil fatty acids
Acid value, mg KOH/g	197.20	202.20	264.70
Iod value, g I/100 g	87.70	128.00	8.81
Composition (% wt)*			
C6:0		—	0.29
C8:0		—	6.28
C10:0		—	5.91
C12:0		—	48.50
C14:0		—	19.10
C16:0		13.95	9.57
C18:0		5.79	1.35
C18:1 w9		30.73	6.42
C18:1 w7		7.46	—
C18:2		42.16	1.37

*The fatty acid composition was determined by capillary gas chromatography under the following conditions: Column, Ultra 2 25 m × 0.32 mm × 0.52 μm film thickness of 5% diphenyl, 95% dimethyl polysiloxane; N_2 carrier gas at 2.5 mL/min; air flow of 374 mL/min; hydrogen flow of 27 mL/min; injection, split 100:1, 0.5 μL; injector temperature, 473 K; oven temperature program, 303 K (5 min), 303–443 K (278 K/min), 443 K (5 min), 443–473 K (276 K/min), 473 K (30 min); flame ionization detector temperature, 523 K.

applied to remove coproduced water from the system, thereby shifting the equilibrium toward ester formation. The stirring rate was adjusted to 1400 rpm, and the pressure was kept at 20 mmHg during the reaction.

Samples were withdrawn at selected time intervals and placed in a 363 K water bath for 15 min to inactivate the enzyme. The samples were centrifuged to separate the pressed seeds, and the oil phase was transferred into a centrifuge tube containing distilled water to separate the glycerol phases and recentrifuged. The oil phase was dried using anhydrous Na_2SO_4. The composition of ester product was investigated by TLC-FID Iatroscan TH-10 analyzer with SIII rods (Iatron Lab. Inc., Tokyo). Complete separation of the lipid mixture was achieved by petroleum ether (bp: 313–333 K): diethyl ether: acetic acid (70:30:2) into triglycerides (TG), fatty acids (FA), 1,3-diglycerides (1,3-DG), 1,2-diglycerides (1,2-DG), 1-monoglycerides (1-MG), and 2-monoglycerides (2-MG). The automatic scanning of rods was performed under the following conditions: flow rate of hydrogen, 160 mL/min; flow rate of air, 2000 mL/min; scan speed, 30 s/scan. A standard mixture including TG, FA, 1,3-DG, 1,2-DG, 1-MG, and 2-MG was used for the evaluation purposes and quantitative determination following the procedure of Ranný (10).

These procedures were conducted to determine whether lipase of the pressed *N. sativa* seeds would catalyze the esterification of fatty acid to

glycerol and the optimum conditions on the enzymatic esterification reaction. Two set of experiments have been carried out. Oleic acid was used for the initial investigation, and the optimum conditions were clearly determined. To investigate the optimum molar ratio of glycerol/fatty acid, the reaction was conducted at ratios of 0.75, 1.0, 1.5, 3.0, and 6.0 of glycerol to oleic acid (a ratio of 1 corresponding to the exact stoichiometry) keeping pressed seeds content at 40% based on total weight and temperature at 318 K. The effect of the amount of the pressed seeds was investigated at optimum molar ratio of glycerol to oleic acid and at 318 K by using 45 and 50% pressed seeds content. To investigate the effect of temperature, the reaction was conducted at 308, 318, 328, and 338 K keeping pressed seeds content and molar ratio at their optimum values. In the second set of experiments, the effect of fatty acid composition was investigated by the esterification of glycerol to sunflower oil fatty acids and coco oil fatty acids at the determined optimum conditions.

RESULTS AND DISCUSSION

Effect of Glycerol–Oleic Acid Ratio

To determine the effect of molar ratio, reactions were conducted at ratios of 0.75, 1.0, 1.5, 3.0, and 6.0 of glycerol to oleic acid (1 is equal to stochiometric ratio) at 318 K keeping pressed seeds content at 40% based on total weight during the 72 h reaction period. The product composition as a function of glycerol/oleic acid ratio and the effect of glycerol/oleic acid mol ratio on the triglyceride synthesis as a function of reaction time are shown in Figs. 1 and 2, respectively. As can be seen, the stochiometric ratio is the optimal molar ratio for the esterification product with the highest triolein concentration (67.67%). This product contains 8.65% diolein, 1.29% monoolein, and 23.02% oleic acid. At the ratio above 1, triolein yield decreases with increasing diolein and monoolein content. These results suggest that desired glyceride composition would be obtained at different molar ratios of glycerol to oleic acid. Similar results were observed by others (*11*).

Effect of Pressed Seed Content

To investigate the effect of pressed seed content, reactions were conducted at stochiometric glycerol/oleic acid molar ratio and 318 K keeping pressed seed content at 40, 45, and 50% based on total weight. The results were presented in Fig. 3. As can be seen, the triglyceride content was increased by increasing pressed seed content. In the reaction conducted at 50% pressed seed content, the conversion rate was decreased. This might

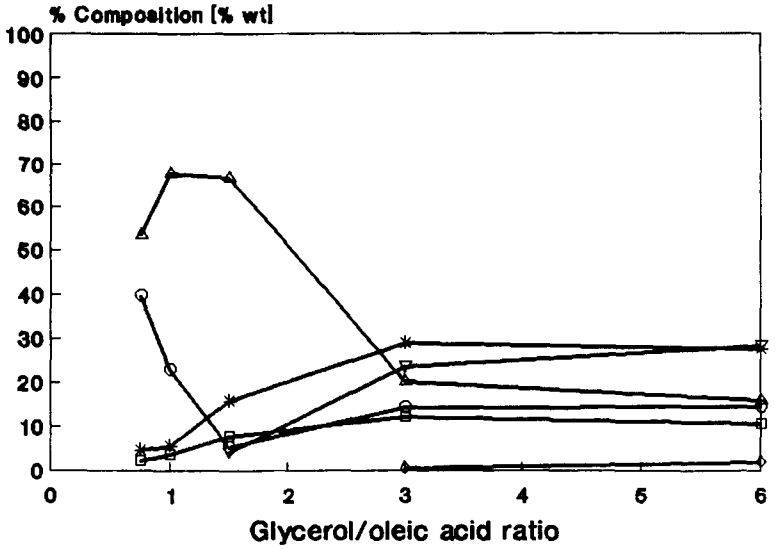

Fig. 1. Comparison of the product composition as a function of glycerol/oleic acid ratio. (Temperature: 318 K; pressed seed content: 40%; 1 =stochiometric ratio) △, TG; o, FA; *, 1,3-DG; □, 1,2-DG; ▽, 1-MG; ◇, 2-MG.

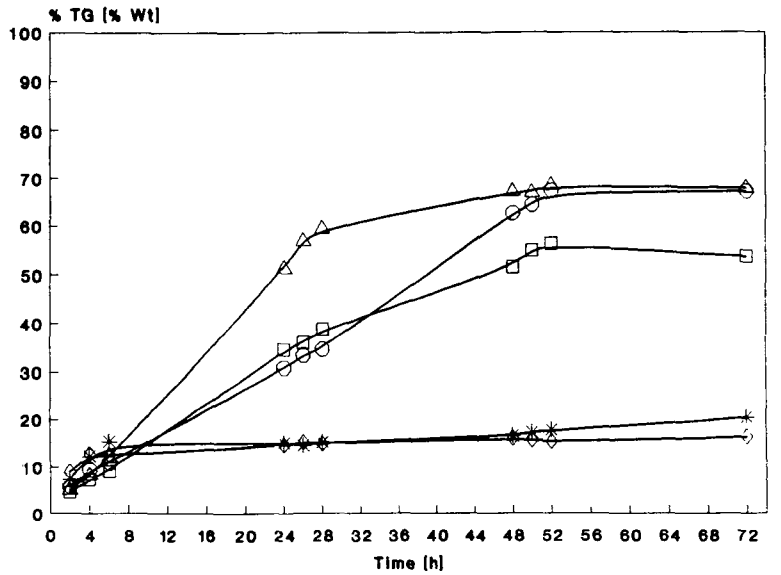

Fig. 2. The effect of glycerol/oleic acid molar ratio on the synthesis of triglyceride as a function of reaction time. (Temperature: 318 K; pressed seed content: 40%.) □, G/OA:1/4; △ G/OA:1/3; o, G/OA:1/2; *, G/OA:1/1; ◇, G/OA:2/1.

be related to the excess solid material in the reaction medium causing difficulty in stirring the reaction media. The best triolein synthesis was observed with the reaction at 45% pressed seed content. The ester product contained 72.82% triolein, 8.20% diolein, and 18.96% oleic acid under these conditions.

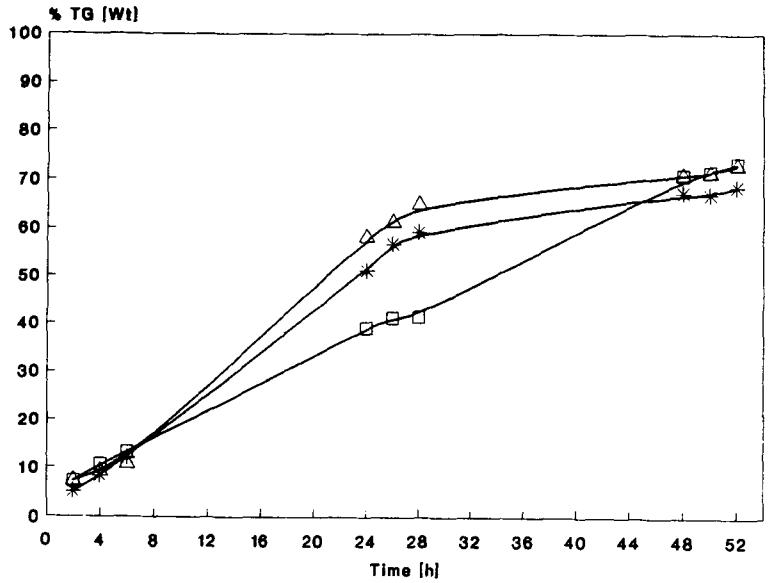

Fig. 3. Comparison of the triglyceride contents of the products that were produced with different lipase content as a function of reaction time. (Temperature: 318 K; G/OA molar ratio: ⅓.) *, 40%; △ 45%; □, 50%.

Effect of Temperature

It was observed that when the pressed seed content was 45% based on total weight and at the stochiometric glycerol/oleic acid molar ratio, the optimum temperature for the esterification of glycerol to oleic acid by native lipase of *N. sativa* seed was 328 K. Under these conditions after 58 h, ester product consists of 81.68% triolein, 5.08% diolein, and 13.24% oleic acid. The effect of temperature on the triolein production is shown in Fig. 4. As can be seen, the increase in process temperature increased the conversion until the lipase activity was affected by high temperature. The conversion decreases if the reaction is conducted at 338 K. This may be related to the loss of lipase activity at this high temperature. In our previous study, where we investigated hydrolysis of used frying oil by the native lipase of *N. sativa* seed, loss of lipase activity was observed above 323 K (*8*). The ester composition of the reaction conducted with 45% pressed seed content, at 328 K and at stochiometric glycerol/oleic acid molar ratio, which shows the highest triolein synthesis, was calculated by using the values determined with the TLC-FID analyzer as shown in Fig. 5. As can be seen, this native lipase prefers the esterification on the sn-1 and sn-3 position of the glycerol, but to keep in view the high triglyceride synthesis observed at the end of the reaction, the native

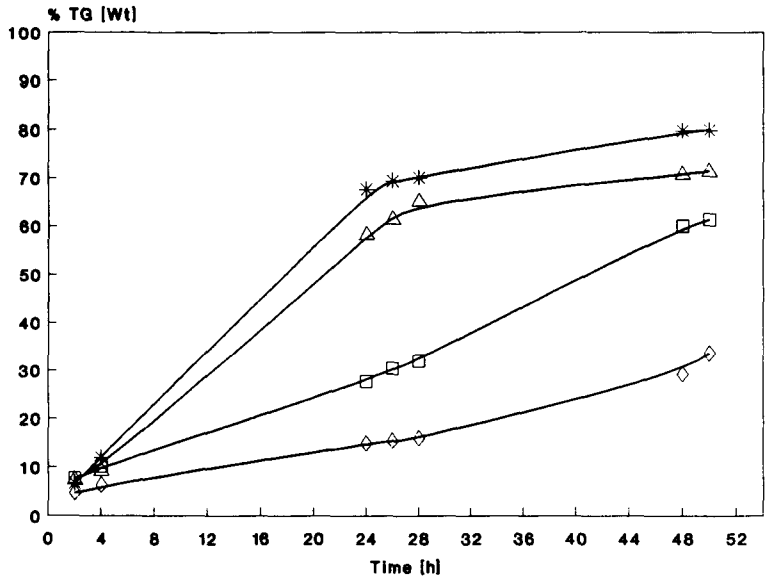

Fig. 4. Comparision of the triglyceride contents of the products that were produced with different temperatures as a function of reaction time. (Pressed seed content: 45%; G/OA molar ratio: ⅓.) ◇, 308 K; △, 318 K; *, 328 K; □, 338 K.

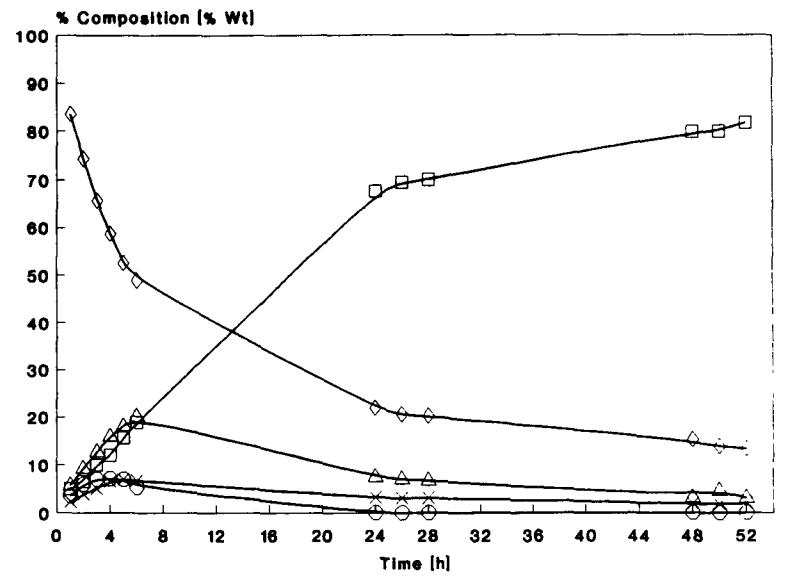

Fig. 5. The product composition from the reaction conducted at optimum conditions. (Temperature: 328 K; pressed seed content: 45%; G/OA molar ratio: ⅓.) □, TG; ◇, FA; △, 1,3-DG; x, 1,2-DG; o, 1-MG.

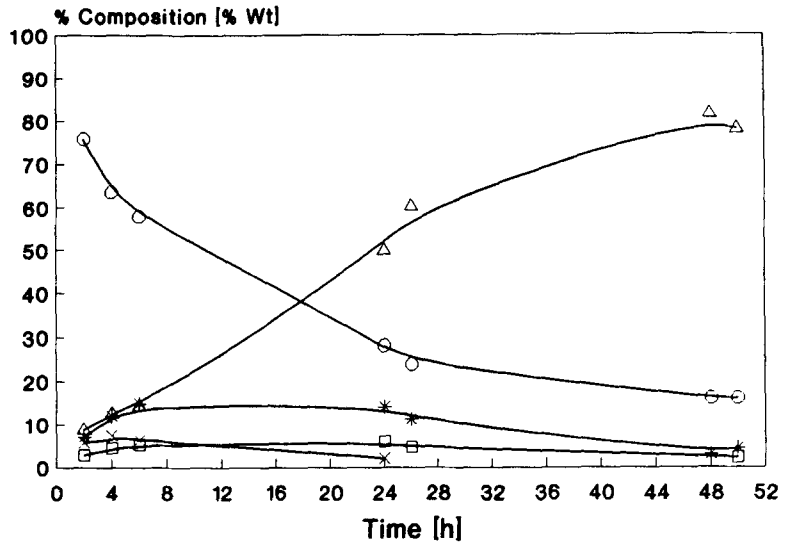

Fig. 6. The product composition from the esterification reaction of glycerol to sunflower oil fatty acids at optimum conditions. (Temperature: 328 K; pressed seed content: 45%; G/FA molar ratio: ⅓.) △, TG; o, FA; *, 1,3-DG; □, 1,2-DG; x, 1-MG.

lipase of *N. sativa* seed can be described as a nonspecific lipase, or the synthesis of the triolein can be explained with the enzyme-catalyzed isomerization of the enzymatically formed 1-MG or 1,3-DG into 2-MG or 1,2-DG, respectively, followed by the esterification of the freed primary hydroxyl group. A similar expression was presented for the triolein synthesis catalyzed by immobilized *Mucor miehei* lipase (1,3-specific) by Lortie and coworkers (*12*)

Esterification of Glycerol with Mixed Fatty Acid

In the first step of study, pure oleic acid was used as the fatty acid to investigate the esterification reaction. However, for industrial applications, it may be convenient to use a fatty acid mixture instead of a pure fatty acid. For this aim and the determination of the effect of fatty acid composition, the esterification of glycerol to fatty acids of sunflower oil and coco oil by native lipase of *N. sativa* seed was investigated at the optimum conditions described above. The reaction conducted at 328 K, 45% pressed seed content, and stochiometric ratio of glycerol to sunflower oil fatty acids yields 77.78% TG, 6.40% DG, and 15.82% FA after 50 h. The product composition is presented in Fig. 6 by using the values determined with TLC-FID analyzer. At the esterification reaction of glycerol with coco oil fatty acids, the conversion of fatty acids was only 9.00% after 50 h. The difference

Table 2
Unreacted Fatty Acid Composition at the Esterification Reaction of Glycerol with Sunflower Oil and Coco Oil Fatty Acids by Native Lipase of Pressed *N. sativa* Seed*

	Fatty acids, % wt			
	Sunflower oil fatty acids		Coco oil fatty acids	
Faty acids	24 h	50 h	6 h	50 h
C6:0	—	—	0.80	0.41
C8:0	—	—	6.07	3.27
C10:0	—	—	5.88	5.52
C12:0	—	—	48.09	49.53
C14:0	—	—	18.40	18.79
C16:0	13.64	13.92	9.53	9.88
C18:0	5.49	5.67	2.59	3.69
C18:1 w9	29.17	28.48	6.79	6.88
C18:1 w7	7.80	8.05	—	—
C18:2	43.90	43.88	1.86	2.01

*The fatty acid composition was determined by CGC under the same conditions described in Table 1.

may be the result of the consistency of the oil. Coco oil consists of saturated fatty acids in a large range of different chain lengths ranging from C:6 to C:18. These results show that the native lipase would have a selectivity for fatty acid chain length and/or saturation degree. To determine the effect of fatty acid chain length and saturation degree, the unreacted fatty acids of sunflower oil and coco oil in the samples tested during the reactions were separated by a suitable procedure (*13*) and converted methyl esters (*14*) were investigated by capillary gas chromatography. These results are shown in Table 2. As can be seen, the native lipase does not have a certain selectivity to sunflower oil fatty acids. Both experiments showed that this native lipase has a preference for long-chain fatty acids as compared to the shorter ones. To determine the effect of saturation degree of fatty acids, the esterification must be carried out with pure fatty acids with a different degree of saturation.

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