Synthesis of Geranyl and Citronellyl Esters of Coconut Oil Fatty Acids Through Alcoholysis by *Rhizomucor miehei* Lipase Catalysis

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ABSTRACT: Synthesis of geranyl and citronellyl esters of mixed fatty acids has been investigated by alcoholysis of coconut oil (CNO) using *Rhizomucor miehei* lipase. CNO fatty acid esters of geraniol and citronellol have unique mild flavors that can be used in food materials. Both geraniolysis and citronellolysis of CNO produce flavor esters in good yield. Depending on substrate concentration the molar yield is more than 50%. The optimized reaction conditions were: pressure, atmospheric; temperature, 50°C; incubation period, 5 h; and Lipozyme, 10 % (w/w).

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Biochemically or chemically catalyzed alcoholysis of vegetable oils to produce fatty acid esters has been investigated for its versatility in commercial applications (1–3). Investigators have used different straight-chain low and high molecular weight alcohols (4). Selective alcoholysis with low molecular weight alcohols (methanol or ethanol) produces biodiesel fuel (5), and with medium and high molecular weight alcohols produces pan release agents, lubricant, and the like (6). The pattern of fatty acid ester utilization is therefore dependent on the alcohol moiety of the ester molecule formed. If these theoretical and practical concepts are applied, flavor esters of mixed fatty acids can be produced by using vegetable oils of choice and terpene alcohols, such as geraniol and citronellol.

Previous reports on the synthesis of terpene esters of monocarboxylic acids involve either chemical (acid) catalysis (7) or enzymatic reactions such as transesterification (8–14) or direct esterification (15–19). Enzymatic synthesis is beneficial not only from an environmental or energy-saving point of view but also for product specificity, especially in synthesizing flavor and fragrance compounds where operating temperature governs product quality. The present study has exploited the enzymatic synthesis of flavor esters with mild, sweet aromas, from mixed fatty acids. Synthesis of esters of mixed fatty acids from triacylglycerols such as coconut oil (CNO), and terpene alcohols has not been reported to date. This paper focuses on the synthesis and optimization of reaction parameters that affect lipase (immobilized *Rhizomucor miehei*)-catalyzed syntheses of flavor esters from CNO.

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MATERIALS AND METHODS

Materials. CNO was purchased from Shalimar Coconut Oil Ltd. (Calcutta, India). Immobilized *R. miehei* lipase (Lipozyme IM), expressed from the corresponding gene cloned into *Aspergillus oryzae*, was a gift from Novo Nordisk A/S (Bagsvaerd, Denmark). The immobilized enzyme preparation had a particle size of 0.2–0.6 mm and a water content of 10% (as reported by Novo). The typical activity of this enzyme is 5–6 batch acidolysis units novo per gram (BAUN/g). Geraniol and citronellol were purchased from the local Calcutta market (purity was greater than 98% as determined by gas–liquid chromatography of the sample). Silica gel G [thin-layer chromatography (TLC) grade] was purchased from Tara Chemicals (Calcutta, India), and silicic acid (60 to 120 mesh) was obtained from Loba Chemie Pvt. Ltd. (Mumbai, India). All other solvents and chemicals used were of analytical grade and were used without further purification.

Characterization of fatty raw materials. CNO was characterized prior to its use. Color (Lovibond, 1-inch cell), peroxide value (meq/kg), saponification value (mg/g), fatty acid composition (%, w/w), and free fatty acid content (%, w/w) were measured following the standard AOCS methods (20).

Alcoholysis method. Geraniolysis and citronellolysis of CNO were carried out in a 25-mL conical flask with B-14 joint. Geraniol or citronellol, plus CNO were taken at varying molar ratios (e.g., 3:1, 2:1, 2:1.5, etc.), and immobilized enzyme *R. miehei* (10%, w/w) was added. The substrate mixture and the enzyme were stirred on a magnetic stirrer with a Teflon-coated bar magnet (1.27 cm long) at 50°C under atmospheric pressure for 5 h. A control with no enzyme was incubated under the same conditions.

Monitoring the reaction. To optimize the time course of reaction, a model reaction (containing CNO and geraniol at a stoichiometric molar ratio of 1:2 plus 10% w/w, enzyme) was monitored by drawing a minute quantity (at a microgram

level, for TLC) of reaction mixture at intervals of 1 h, so that the total quantity did not change appreciably and thereby affect the equilibrium of the reaction. After removal of enzyme by filtration, the product mixtures were subjected to TLC to estimate the time for equilibrium conversion. The yields of the sample product-esters drawn were then measured through isolation of the pure esters by column chromatography.

Extraction and analysis of reaction products. At the end of the reaction period of 5 h, the mixture was filtered under vacuum (30 mm Hg pressure) to remove Lipozyme, which was washed with petroleum ether (b.p. 40 to 60°C) until it was free from product mixture. The recovered Lipozyme was stored in a refrigerator until next use. The crude reaction product was dried over anhydrous sodium sulfate and then subjected to column chromatography.

Qualitative analyses were carried out by TLC on glass plates (20 × 20 cm), coated with a 0.2-mm layer of silica gel G. Plates were spotted with the same amount of reaction product drawn at different times of incubation and developed in 100 mL of *n*-hexane/diethyl ether (90:10, vol/vol). The spots were then visualized by iodine absorption. Appearance of ester spots of nearly the same area and intensity indicates that the reaction reached completion.

Quantitative isolation of product esters. Geranyl and citronelloyl esters formed were separated by passing a known quantity of the product mixtures through a bed of silicic acid (12 g) packed into a glass column (15 × 1.5 cm). The mixture was eluted first with 75 mL hexane and then with 150 mL of *n*-hexane/diethyl ether (99:1, vol/vol) to isolate the pure ester. The purity of the products was further confirmed by TLC, where the appearance of a single spot in the purified sample indicated that the column-separated product was pure.

TABLE 1
Effect of Reaction Time on *Rhizomucor miehei* Lipase-Catalyzed Geraniolysis of Coconut Oil[a]

Time of reaction (h)	Percent molar conversion of	
	Oil	Alcohol
2	40.21	40.16
4	68.53	68.50
5	77.78	77.27
6	78.31	78.40

[a]Reactions were carried out at 50°C, under atmospheric pressure, using 10% w/w enzyme, and an oil/alcohol ratio of 1:2.

Calculation of the percentage conversion. The percentage conversion of both the oil and alcohol has been calculated by the following formula:

$$\text{molar conversion of oil (or alcohol) into ester (\%)} = \frac{\text{mmol ester formed}}{\text{theoretical maximum possible number of mmol of ester from oil (or alcohol)}} \times 100 \quad [1]$$

RESULTS AND DISCUSSION:

The CNO sample used for the synthesis of terpene esters was an authentic one as revealed by its saponification value (259 mg/g), and fatty acid composition ($C_{6:0}$-1.3, $C_{8:0}$-5.7, $C_{10:0}$-9.2, $C_{12:0}$-45.3, $C_{14:0}$-17.0, $C_{16:0}$-7.4, $C_{18:0}$-4.1, $C_{18:1}$-9.2, $C_{18:2}$-0.8, % w/w). The high quality of the sample was supported by its content of free fatty acid (0.12 % w/w), color (in Lovibond scale, 3Y+0.1R) and the peroxide value (3.1 meq/kg).

Time course. Time course studies indicated the performance of the enzyme as well as the progress of reaction, and

TABLE 2
Effect of Enzyme Concentration on the Geraniolysis and Citronellolysis of Coconut Oil[a]

Alcohol used	Oil/alcohol molar ratio	Enzyme concentration (% w/w)	Percent molar conversion of	
			Oil	Alcohol
Geraniol	1:2 (stoichiometric)	5.0	63.07	62.66
		7.5	69.12	68.80
		10.0	77.78	77.27
Citronellol	1:2 (stoichiometric)	5.0	79.87	79.87
		7.5	82.53	82.40
		10.0	89.29	89.28

[a]Reactions were carried out for 5 h at 50°C, under atmospheric pressure.

TABLE 3
Effect of Temperature on the Geraniolysis and Citronellolysis of Coconut Oil[a]

Alcohol used	Oil/alcohol molar ratio	Temperature (°C)	Percent molar conversion of	
			Oil	Alcohol
Geraniol	1:2 (stoichiometric)	50	77.78	77.27
		40	70.06	70.13
		30	68.54	68.48
Citronellol	1:2 (stoichiometric)	50	89.29	89.28
		40	74.51	74.38
		30	72.10	72.30

[a]Reactions were carried out for 5 h under atmospheric pressure using 10% w/w enzyme.

helped to determine the shortest time necessary to obtain good yields and thereby make the process cost-effective.

Table 1 shows the effect of time on the percentage molar conversion of CNO to geranyl esters of CNO fatty acids. These data suggest that a reaction period of 5 h is optimal for the conversion.

Effect of enzyme concentration. The amount of lipase is a crucial economic factor for any bioconversion process. For industrial requirements the concentrations of lipases reported are often too high (21). Enzyme concentrations of 83 and even 93% (w/w) have been reported (22,23), which are unsuitable commercially.

From Table 2 it is evident that if CNO and either geraniol or citronellol (at a molar stoichiometric ratio of 1:2) are used, then 7.5% (w/w) enzyme results in molar conversions of 68.8 and 82.4% with respect to geraniol and citronellol, respectively, and molar conversions with respect to oil are 69.12 and 82.53% when geraniol and citronellol are used in respective cases. However, under the same conditions listed above, if 10% (w/w) lipase is used, which is the most commonly used percentage for biochemical reactions, 77.78 and 77.27% conversions are achieved with respect to oil and geraniol. Moreover, 89.29 and 89.28% conversions are achieved with respect to oil and citronellol when citronellolysis is carried out.

Effect of temperature. To observe the effect of temperature, reaction of CNO with geraniol (using stoichiometric amount) has been carried out at three different temperatures (30, 40, and 50°C). The results (Table 3) indicate that of the three temperatures studied 50°C is preferable in terms of the percentage conversion with respect to both terpene alcohol and oil into ester.

Effect of pressure. Reduction in the percentage molar conversion of CNO into gerany esters was observed if the reaction was carried out below atmospheric pressure (such as 400 or 10 mm Hg) at 50°C (Table 4). This may have resulted from increments in vapor pressure of low molecular weight fatty acid esters at very low pressure with the subsequent removal of some low molecular weight fatty acid esters.

Effect of substrate concentration. The effects of terpene alcohols and CNO concentration on the alcoholysis reaction were investigated, since previous findings suggest that both citronellol and geraniol indiscriminately inhibit *R. miehei* lipase (12,15).

From the effect of substrate (CNO vs. geraniol and citronellol) ratios (Table 5), it appears that use of one substrate in excess may raise the percentage molar conversion in terms of the other substrate. This may be due to acyl migration of fatty acids from the 2-position to 1- and 3-positions followed by further alcoholysis. The ability of Lipozyme to act on secondary glyceride esters may also be another explanation for higher conversion. However, the overall results indicate that use of a stoichiometric ratio of oil and alcohol is quite acceptable in terms of conversion.

Effect of enzyme reuse. Reuse of the catalyst is one of the main advantages of using immobilized enzymes. Immobilization allows for the cost of expensive enzymes to be recovered and for reuse in a system, thereby decreasing the process costs and permitting continuous automated production (24). In the present study a negligible loss of activity (5 %) was observed after three consecutive runs, which indicates the feasibility of enzyme recycling in this synthesis.

The overall study indicates that CNO terpenyl esters can be synthesized by enzymatic alcoholysis at atmospheric pressure in good yield.

Table 4
Effect of Pressure on the Geraniolysis of Coconut Oil[a]

Pressure (mm Hg)	Percent molar conversion of	
	Oil	Alcohol
10	66.99	67.00
400	74.03	74.00
760	77.78	77.27

[a] Reactions were carried out for 5 h at 50°C temperature using 10% w/w enzyme.

TABLE 5
Effect of Substrate Ratios on the Geraniolysis and Citronellolysis of Coconut Oil[a]

Alcohol used	Molar ratio of oil/alcohol	Percent molar conversion of	
		Oil	Alcohol
Geraniol	1:2	77.78	77.27
	1:3	113.72	75.00
	1.5:2	65.12	98.53
	1:1	46.22	97.82
Citronellol	1:2	89.29	89.28
	1:3	120.91	85.74
	1.5:2	63.56	98.41
	1:1	49.50	98.20

[a]Reactions were carried out at 50°C and 760 mm Hg pressure for 5 h, using 10% w/w enzyme.

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