Synthesis of Monoterpene Esters by Alcoholysis Reaction with *Mucor miehei* Lipase in a Solvent-Free System

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ABSTRACT: The syntheses of geranyl acetate and citronellyl acetate by alcoholysis reaction catalyzed by immobilized lipase from *Mucor miehei* was studied for the first time in a solvent-free system. Reactions were carried out at a terpene alcohol/acyl donor molar ratio of 1:5 with Lipozyme at 10% of the total weight of the reactants in a solvent-free system. Incubations were carried out at 55 to 60°C for ethyl and butyl acetates as acyl donors, whereas for methyl acetate the incubation temperature was 40 to 45°C. Excess concentration of acyl donor increases the percentage of geranyl acetate and citronellyl acetate, while excess of terpene alcohol concentration decreases the same. Yields from 75 to 77% molar conversion (90 to 98% conversion, w/w) were obtained after 8 to 28 h of reaction time. *JAOCS 75,* 651–655 (1998).

KEY WORDS: Citronellol, citronellyl acetate, geraniol, geranyl acetate, immobilized lipase, *Mucor miehei,* transesterification.

Short-chain monocarboxylic acid esters of terpene alcohols are important fragrance compounds, generally synthesized by the direct addition of a lower organic acid to terpenic olefins under nonaqueous conditions in the presence of an acid catalyst or a strong cation exchange resin (1). The esters of terpene alcohols are also synthesized by direct esterification of terpene alcohols with short-chain acids by chemical methods. In recent years, enzymatic syntheses with lipases (triacylglycerol hydrolases E.C. 3.1.1.3) as catalysts have been used to produce a number of commercially important flavor esters in anhydrous organic solvents (2–6), both by transesterification (7–13) and by direct esterification (3–5,14,15).

The nature of the acyl donor used plays an important role in the transesterification or esterification methodologies in the presence of lipase as catalyst. Thus, yields of geranyl acetate and citronellyl acetate were low when acetic acid was used as an acyl donor in both transesterification and esterification reactions (14,16,17). A distinct reduction in the pH of the microenvironment of the lipases by acetic acid actually interfered with their aqueous layer (18,19). Moreover, the yield of some geranyl esters by direct esterification with an immobilized lipase was also low (20). These problems have been overcome by lipase-catalyzed transesterification reactions with other acyl donors, such as acetic anhydride (21), isopropenyl acetate (8), and triacetin (8,12). Chulalaksananukul *et al*. (11) studied the lipase-catalyzed transesterification of geraniol with C_1, C_2, C_3, C_4, and higher acetates as acyl donors. The reactions were carried out in the presence of organic solvents because the solvent hydrophobicity influenced enzymatic activity (22). Yee and Akoh (21) reported quite high yields for the *Pseudomonas* sp. lipase-catalyzed transesterification of geranyl acetate in the presence of organic solvents (petroleum ether, hexane, and pentane) of log *P* values ≥3.0 (log *P* is the partition coefficient between water and octanol).

The alcoholysis reaction of terpene alcohols and esters of short-chain acids and alcohols to make terpene alcohol esters has not been studied adequately. Two important points need to be considered in the synthesis of terpene alcohol esters by alcoholysis reactions. Because the acetates act as solvents, it becomes imperative to examine whether the alcoholysis reaction can be conducted without using any hydrocarbon solvents. Oguntimein *et al*. (23) synthesized geraniol esters by direct esterification in a solvent-free system with *Candida antarctica* lipase. This paper presents alcoholysis reactions with *Mucor miehei* lipase in the absence of any organic solvent, which, to the best knowledge of the authors, have not been reported before. We also present here an isolation process for the recovery of the terpene alcohol esters in pure form. These two aspects are important for commercial synthesis of terpene alcohol esters.

The present study deals with the synthesis of acetate esters of geraniol and citronellol by lipase-catalyzed alcoholysis in a solvent-free system and investigates the effects of substrate concentration (terpene alcohols/acyl donor molar ratio), reaction temperature, and reaction period.

MATERIALS AND METHODS

Materials. All alcohols, esters, and solvents were commercially available and were used without further purification. The immobilized lipase from *M. miehei* (Lipozyme IM) was obtained from Novo-Nordisk A/S (Bagsvaerd, Denmark). Silicic acid (60 to 120 mesh) for column chromatography was

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purchased from Loba Chemicals (Calcutta, India). Silica gel G for thin-layer chromatography (TLC) was purchased from Tara Chemicals (Calcutta, India). Deuterated chloroform ($CDCl_3$) for ^1H NMR spectrometry was purchased from Aldrich Chemicals Co. Ltd. (Gillingham, United Kingdom).

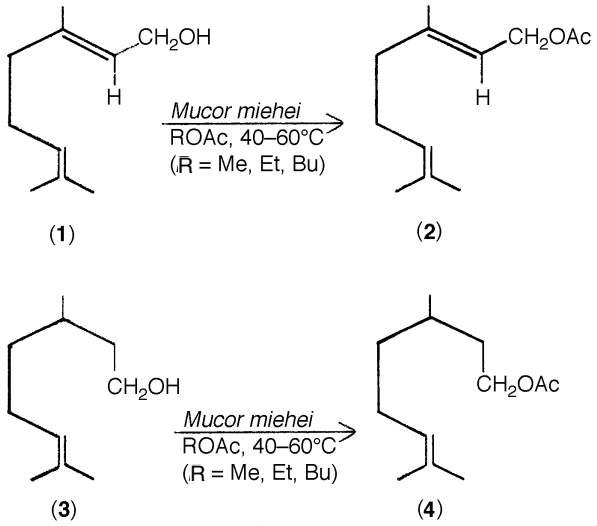
Alcoholysis method. Ester synthesis was carried out by magnetically stirring the reactants in a 50-mL standard-joint conical flask, fitted with a condenser and a $CaCl_2$ guard tube. With methyl acetate, the reaction was carried out at varying alcohol/ester molar ratios (1:1, 1:3, and 1:5) with immobilized *M. miehei* lipase (Lipozyme) at 10% of the total weight of the reactants. The weight of terpene alcohols was always kept constant, and only the weight of acyl donors was varied. Incubations were carried out at 55 to 60°C when ethyl acetate and butyl acetate were used as acyl donors. The incubation temperature was kept between 40 and 45°C with methyl acetate. The optimal alcohol/ester molar ratio for use with methyl acetate was used in the reaction involving ethyl and butyl acetates as acyl donors.

Extraction and analysis. At the end of the incubation period, each reaction mass was filtered under vacuum to remove the Lipozyme. The enzyme-free material from the methyl acetate reaction product was extracted with distilled water (3 × 15 mL) to remove unreacted methyl acetate and methanol. The water-washed organic phase, which contained the terpene ester, was next dried over anhydrous sodium sulfate. The dried material was then subjected to column chromatography on silicic acid (60 to 120 mesh) for quantitative isolation of the esters of terpene alcohols.

After the reactions with ethyl acetate and butyl acetate, the Lipozyme was separated by vacuum filtration. The reaction products were next subjected to vacuum distillation to remove the unreacted acyl donors and the liberated alcohols, *viz*., ethanol and butanol. The completeness of the removal of acyl donors was tested by gas–liquid chromatography (HP 5890A; Hewlett-Packard, Avondale, PA), equipped with a flame-ionization detector. A 10% DEGS (Hewlett-Packard) column was used. Oven, injector, and detector temperatures were 140°C (isothermal), 200°C, and 210°C, respectively. The carrier gas was nitrogen (flow rate 30 mL/min).

The crude products, free from acyl donors, were then subjected to column chromatography on silicic acid (60 to 120 mesh) with 100 mL *n*-hexane/diethyl ether (99:1, vol/vol). The solvents were then evaporated on a water bath, followed by applying vacuum at ambient temperature (30°C) to give pure geranyl and citronellyl acetates as colorless oils (Scheme 1). The purity of the products was confirmed by TLC and by using spectral methods, *viz*., Fourier transform infrared (FTIR) spectroscopy and proton nuclear magnetic resonance (^1H NMR) spectrometry.

Qualitative analyses were carried out by TLC. Samples were diluted 1:5 (vol/vol) with chloroform, and the diluted samples were used for TLC analysis on glass plates (20 × 20 cm) with a 0.2-mm layer of silica gel G. The plates were developed with *n*-hexane/diethyl ether (70:30, vol/vol). All spots were identified by strong iodine absorption. The R_f values of the esters were 0.92 for geranyl acetate and 0.90 for citronellyl acetate, respectively.



SCHEME 1

The infrared (FTIR) spectra were recorded on a Perkin-Elmer 1600 Fourier transform spectrometer (Norwalk, CT) with the samples spread between NaCl plates. The ^1H NMR spectra were determined in deuterated chloroform with a Bruker AM-300L spectrometer (Fallenden, Switzerland) that was operated at a frequency of 300 MHz with tetramethyl silane (TMS) as internal standard.

Quantitation. Geranyl acetate and citronellyl acetate were quantitated by standard column chromatographic techniques on silicic acid (60 to 120 mesh). The percentage yields of esters (molar yield percentage) with respect to alcohols for different incubation times were calculated from the weight of esters produced and the weight of alcohols used in the reactions, as well as from their respective molecular weights.

RESULTS AND DISCUSSION

Identification of products by spectral method. The spectral data of the starting alcohols and the product esters are shown in Table 1. The FTIR spectra of geraniol and citronellol showed absorption of hydroxyl groups at 3334 and 3333 cm^{-1}, respectively. The FTIR of the acetates of geraniol and citronellol lacked absorption for the hydroxyl group at 3333 to 3334 cm^{-1} but showed absorption at 1741 cm^{-1}, as expected for the ester carbonyl group. Similarily, from the ^1H NMR data, the resonance signal at δ 3.73 ppm for geraniol and δ 3.68 ppm for citronellol, which are due to methylene protons ($–CH_2$) of the $–CH_2OH$ end group, disappeared in the acetates and were replaced by δ 4.57 ppm for geranyl acetate and δ 4.55 ppm for citronellyl acetate. The increase in δ values in the products, compared to the starting alcohols, is due to formation of the acetate ($–OCOCH_3$) group, which shifts the methylene protons to higher frequency (24).

The FTIR and ^1H NMR data were compared with the stan-

TABLE 1
Spectral Data of Geraniol, Geranyl Acetate, Citronellol , and Citronellyl Acetate[a]

Name of compound	FTIR ν_{max} (cm^{-1})[a]	^1H NMR [δ (ppm); *J* (Hz)]	Mol. wt. (M^+)
Geraniol	3334, 2958, 2924, 1458, 1373, 1057	5.47 (*t*, 1H, *J* = 6) 5.15 (*t*, 1H, *J* = 6) 4.2 (*d*, 1H), 3.73 (*q*, 2H), 2.08 (*m*, 4H), 1.77 (*s*, 6H), 1.64 (*s*, 3H)	154
Geranyl acetate	2924, 2365, 1741, 1447, 1374, 1235, 1024	5.39 (*t*, 1H, *J* = 6) 5.06 (*t*, 1H, *J* = 3) 4.57 (*d*, 2H, *J* = 6), 2.1 (*s*, 3H), 2.03 (*m*, 4H), 1.69 (*s*, 6H), 1.62 (*s*, 3H)	196 (C:H:O 73.31: 10.02: 16.52)
Citronellol	3333, 2958, 2924, 1456, 1377, 1057, 1008	5.13 (*t*, 1H, *J* = 6) 4.13 (*q*, 1H), 3.68 (*m*, 2H), 2.61(*m*, 2H), 2.02 (*m*, 2H), 1.71 (*s*, 6H), 1.60 (*m*, 1H), 1.40 (*m*, 2H), 0.91 (*m*, 3H)	156
Citronellyl acetate	2957, 2361, 1741, 1452, 1373, 1238, 1035, 829	5.06 (*t*, 1H, *J* = 6) 4.55 (*t*, 2H, *J* = 8) 2.02 (*m*, 2H), 2.00 (*s*, 3H), 1.65 (*s*, 6H), 1.55 (*br s*, 1H), 1.30 (*m*, 4H), 0.89 (*m*, 3H)	198 (C:H:O 70.60: 10.44: 18.64)

[a]FTIR, Fourier transform infrared; NMR, nuclear magnetic resonance.

dard samples, which conclusively indicate the formation of geranyl and citronellyl acetates by alcoholysis.

Substrate molar ratio. The effect of substrate concentration on the synthesis of flavor esters with *M. miehei* has been reported (25). Inhibitory effect of geraniol on *M. miehei* lipase (IM 20) activity was observed by Chulalaksananukul *et al*. (11) and by Claon and Akoh (3), although the degree of inhibition depends mainly on the type of enzyme used (26).

Initially, we studied the alcoholysis reaction at 1:1 and 1:3 terpene alcohol/ester (methyl acetate) molar ratios, and geranyl acetate and citronellyl acetate were obtained in poor yields. The molar percentage conversions of geraniol acetate were 16.54 and 19.25% for geraniol/methyl acetate molar ratios of 1:1 and 1:3, respectively, whereas for citronellyl acetate, the molar conversions were 23.85 and 25.25%, even after 8 h of incubation. By increasing the terpene alcohol/ester molar ratio to 1:5, the yield became as high as 77.92 mol% after 8 h of incubation (Table 2). Hence, for both monoterpene esters yields improved as the concentration of acyl donors was increased and that of terpene alcohol was decreased.

Time course of reaction. The time course of lipase-catalyzed synthesis of geranyl acetate and citronellyl acetate is shown in Table 2. About 42% geraniol and 44% citronellol conversions to acetates were attained in 4 h by using a molar ratio of 1:5 for terpene alcohol/acyl donor (methyl acetate). However, in the present study, substantial conversion was achieved in 8 h for both geranyl acetate and citronellyl acetate by immobilized lipase from *M. miehei*. The lipase-catalyzed alcoholysis of geraniol with propyl acetate as acyl donor, which is regarded as the best acyl donor for geranyl acetate synthesis, has resulted in 85% yield after 3 d of incubation (11). Langrand *et al.* (17) reported the yield for the same reaction, with isoamyl acetate as the substrate, to be 24% after 24 h of incubation. The alcoholysis reactions, when conducted for longer periods, *viz.*, 18 and 28 h, did not increase the yield of the products, but showed a slight decline. We concluded that the highest yields of geranyl acetate and citronellyl acetate could be achieved in 8 h, which would make the process attractive and hence commercially exploitable.

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TABLE 2
Yield in Molar Percentage of Esters by Lipase-Catalyzed Alcoholysis Reactions of Geraniol and Citronellol with Different Acyl Donors

Alcohol (x)	Acyl donor (y)	Molar ratio (x:y)	Reaction temperature (°C)	Reaction time (h)	Conversion[a] (mol%)
Geraniol	Methyl acetate	1:1	40 to 45	8	16.54
		1:3		8	19.25
		1:5		4	42.44
		1:5		8	76.43
		1:5		18	76.07
Geraniol	Ethyl acetate	1:5	55 to 60	8	76.62
				18	75.97
				28	75.32
Geraniol	Butyl acetate	1:5	55 to 60	8	77.14
				18	76.22
				28	71.07
Citronellol	Methyl acetate	1:1	40 to 45	8	23.85
		1:3		8	25.25
		1:5		4	44.25
		1:5		8	77.92
		1:5		18	76.51
Citronellol	Ethyl acetate	1:5	55 to 60	8	77.48
				18	76.11
				28	75.68
Citronellol	Butyl acetate	1:5	55 to 60	8	76.52
				18	77.11
				28	76.84

[a]Conversion = (wt. of terpene ester obtained × M.W. of terpene alcohol)/(initial wt. of terpene alcohol × M.W. of terpene ester) × 100.

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