Lipase-Catalyzed Synthesis of Primary Terpenyl Acetates by Transesterification: Study of Reaction Parameters

Paul A. Claon and Casimir C. Akoh*

Department of Food Science and Technology, Food Science Building, The University of Georgia, Athens, Georgia 30602-7610

Transesterification of geraniol and citronellol with triacetin catalyzed by *Candida antarctica* lipase, SP435, was investigated. The reaction was carried out in *n*-hexane containing 0.1 M terpene alcohol, 0.03 M triacetin, and 10% (w/w reactants) lipase. Time course, effect of added water, temperature, solvent type, and lipase reuse were studied. Yields greater than 96% molar conversion were obtained after 10 h of incubation. Optimum yields were obtained at temperatures between 30 and 40 °C with no added water. Yields ranging from 62 to 97% were obtained for solvent-free synthesis and for solvents with log *P* value ≥0.85. The yields of citronellyl acetate were greater than those of geranyl acetate. The lipase remained active after 17 times of reuse.

Keywords: *Transesterification; organic solvent; primary terpenyl acetates; Candida antarctica; lipase*

INTRODUCTION

Terpene alcohols and their esters are major components of essential oils, a family of lipidic compounds well-known for their organoleptic properties (Croteau, 1980). The ability of lipases to catalyze the synthesis of important terpene esters by direct esterification in biphasic and microaqueous systems has been demonstrated (de Castro et al., 1992; Iwai et al., 1980; Langrand et al., 1988, 1990; Marlot et al., 1985). Few studies have focused on the production of terpene esters by lipase-catalyzed transesterification reactions (Chulalaksananukul et al., 1992, 1993; Gray et al., 1990; Langrand et al., 1988). Although triacylglycerols are the preferred substrates for lipases (Macrae, 1983), they have been used in only one study dealing with lipase-catalyzed synthesis of terpene esters and with relatively low yields (Gray et al., 1990). Lipase-catalyzed transesterification reactions have also been used in the production of partial acylglycerols (Akoh, 1993; Akoh et al., 1992), tailored fats (Bloomer et al., 1990; Kanasawud et al., 1992; Macrae, 1983), and biosurfactants (Chopineau and McCafferty, 1993; Mutua and Akoh, 1993). Our previous study dealt with the reaction parameters affecting the synthesis of terpenyl acetates by direct esterification in organic media (Claon and Akoh, 1993). Although yields greater than 95% were obtained, the inhibitory effect of acetic acid on lipase esterification activity was observed. Transesterification reactions have been proposed to circumvent such effects (Chulalaksananukul et al., 1992; Gray et al., 1990; Welsh et al., 1989). This paper presents a study of the reaction parameters affecting the transesterification of citronellol and geraniol with triacetin as the acyl donor.

MATERIALS AND METHODS

Materials. Nonspecific *Candida antarctica* lipase cloned into *Aspergillus oryzae* and immobilized on polyacrylic resin, SP435 (7000 PLU/g), was provided by Novo Nordisk Bioindustrials, Inc. (Danbury, CT). Geraniol (99% pure), DL-citronellol (95% pure), and triacetin (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO). Pyridine (purified) was obtained from Matheson Coleman & Bell (Cincinnati, OH). All other solvents were of HPLC grade and purchased from Fisher Scientific (Norcross, GA). The reagent for coulometric determination of water (Hydranal-Coulomat AG) was purchased from Crescent Chemical Co., Inc. (Hauppauge, NY).

Transesterification Method. In a typical solvent synthesis of primary terpenyl acetate, 0.1 M terpene alcohol, 0.03 M triacetin, and 10% (w/w reactants) lipase were added to 2 mL of hexane. The samples were incubated in an orbital shaking water bath at 30 °C and 200 rpm for 10 h along with their respective controls (samples without lipase). All of the experiments were run in duplicate. Reaction products of solvent-free synthesized were dissolved in 2 mL of hexane after incubation.

Effect of Solvents. Various solvents were tested as the transesterification medium following the method outlined above. Their water content was measured using a 684 KF coulometer equipped with a 649 stirrer (Brinkman Instruments, Inc., Westbury, NY). A solvent-free sample was also prepared in duplicate and incubated under the conditions described above.

Extraction and Analysis. At the end of the incubation period, the reaction mixtures were cooled in ice and passed through an anhydrous sodium sulfate (Na_2SO_4) column (0.5 × 3 cm) to remove the enzyme and any residual water. Two hundred micrograms of internal standard (± linalool) was added to each sample. Both reactants and product were completely resolved from the internal standard as determined by gas–liquid chromatography (GLC). A 1 μL aliquot was analyzed by GLC in a splitless mode with a Hewlett-Packard HP5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector (FID). A DB-5 fused silica capillary column (30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA) was used and operated isothermally at 150 °C. Injector and detector temperatures were set at 250 and 260 °C, respectively. Helium was used as the carrier gas at a total flow rate of 24 mL/min. Since no side reaction was detected, the extent of synthesis was determined from the amount of terpene alcohol consumed in the reaction and quantified by an on-line computer as previously described (Claon and Akoh, 1993).

For enzyme reuse, the reaction product was removed and passed through an anhydrous sodium sulfate column while the enzyme in the test tube was rinsed with hexane, the solvent evaporated, and the enzyme subsequently dried in a desiccator prior to reuse.

* Author to whom correspondence should be addressed [telephone (706) 542-1067; fax (706) 542-1050].

0021-8561/94/1442-2349$04.50/0

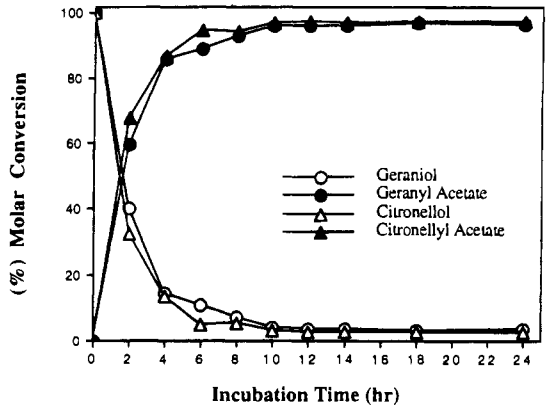

Figure 1. Time course of enzymatic synthesis of geranyl and citronellyl acetate by transesterification in *n*-hexane using *C. antarctica* lipase, SP435.

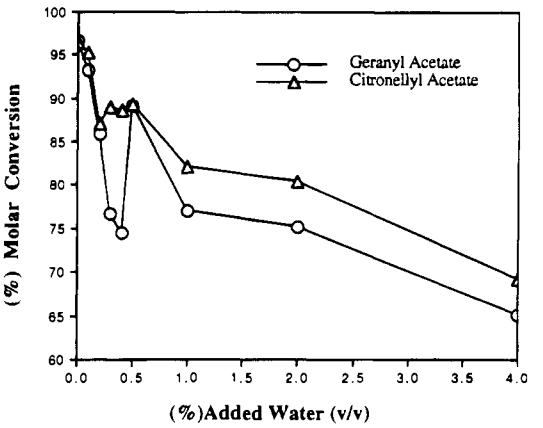

Figure 2. Effect of added water on the transesterification of geraniol and citronellol with triacetin using *C. antarctica* lipase, SP435.

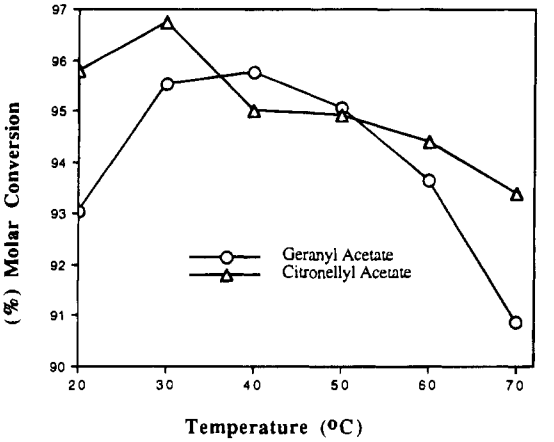

Figure 3. Effect of temperature on the SP435 lipase-catalyzed synthesis of geranyl and citronellyl acetate by transesterification in *n*-hexane.

RESULTS AND DISCUSSION

Time Course. We previously studied the time course of SP435 lipase-catalyzed direct synthesis of citronellyl acetate and found that a yield of 98.2% was obtained after 14 h of incubation. We also observed a loss in the lipase esterification activity at longer incubation periods (data not shown). The time course of the synthesis of geranyl and citronellyl acetate by transesterification reaction using the same lipase showed that conversion yields of 96 and 97%, respectively, were obtained after 10 h of incubation (Figure 1). No loss of lipase activity was observed with prolonged incubation. This suggests that triacetin does not inhibit the enzyme and may be a better acyl donor than acetic acid under the reaction conditions used in the study. Chulalaksananukul et al. (1992) reported a yield of 85% geranyl acetate after 3 days of incubation with propyl acetate as the acyl donor and *Mucor miehei* lipase, IM20, as biocatalyst. On the basis of the results obtained from the time course, a 10 h incubation period was used in the subsequent experiments.

Effect of Added Water. In transesterification, hydrolysis precedes esterification reaction (Yamane, 1987). In organic solvents, the rate of hydrolysis of triacylglycerols is increased (Mukherjee, 1990). The effect of water on lipase-catalyzed synthesis of terpene esters has been reported (Chulalaksananukul et al., 1992; de Castro et al., 1992; Iwai et al., 1980). During the transesterification of geraniol by propyl acetate in *n*-hexane, addition of water to the solvent led to an increase in reaction velocity with an optimum at 3 μL of added water (Chulalaksananukul et al., 1992). Figure 2 shows the effect of water added to the reaction mixture on the transesterification yields. The lipase used (SP435) was more susceptible to increasing water content in the geraniol than in the citronellol samples. The exact reason for the low incorporation in the case of geraniol at below 0.5% added water is unclear at this point. The fact that the highest yield (97%) was obtained for both terpene alcohols when no water was added suggests that the difference in original water content did not adversely affect the lipase activity. However, as the water content increased, the lipase activity in the geraniol samples decreased because of the relatively high original water content in geraniol. At 4% (v/v) added water, the yield of geranyl acetate dropped 33% and that of citronellyl acetate, 29% (Figure 2). At 10% added water, 52 and 45% drops in transesterification yields were observed for geranyl and citronellyl acetate, respectively (data not shown). For both terpene alcohols, the lipase showed partial restoration of transesterification activity at 0.5% added water. Notably, 0.5% added water seemed to be the critical water content required by the enzyme to give acceptable yields of geranyl and citronellyl acetate, beyond which there was a rapid decrease in transesterification activity.

Effect of Temperature. The effect of temperature on lipase activity have been investigated (Akoh et al., 1992; Chulalaksananukul et al., 1992, 1993; Hirata et al., 1990; Novo Industri, 1986, 1992). Temperature effects on lipase-catalyzed ester synthesis were reported to be dependent on the reaction medium, enzyme source, and substrate (Welsh et al., 1989). The transesterification activity of *C. antarctica* lipase, SP435, was monitored at temperatures ranging from 20 to 70 °C. The highest yields were 97.6% citronellyl acetate and 95.3% geranyl acetate at 30 and 40 °C, respectively (Figure 3). In the synthesis of citronellyl acetate by direct esterification, the highest yield (98.6%) was obtained at 20 °C (Claon and Akoh, 1994). At 70 °C, 5 and 3% drops in yield were observed for geranyl and citronellyl acetate, respectively. This difference might be explained by the higher water content in geraniol samples (see Effect of Added Water). Thermal denaturation of enzymes proceeds through a series of reactions that require water (Klibanov, 1986).

Effect of Enzyme Reuse. Enzyme reuse in bioprocesses is of crucial economic importance. *C. antarctica* lipase, SP435, was capable of maintaining esterification

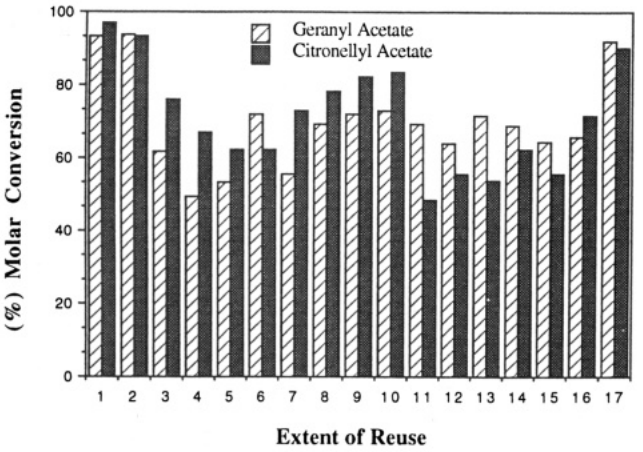

Figure 4. Effect of SP435 lipase reuse on the synthesis of geranyl and citronellyl acetate by transesterification in *n*-hexane.

activity after the 10th run in a previous study involving the synthesis of citronellyl acetate by direct esterification (Claon and Akoh, 1994). The stability of the same lipase under the conditions of our transesterification reactions was tested. After each run, the lipase was washed with hexane and the solvent evaporated. A drop in yield, as much as 34%, was observed after the third run (Figure 4). The yields afterward became erratic. A persistent jelly-like substance was observed at the bottom of the test tube. When the lipase was washed with 10% $NaHCO_3$, followed by hexane, and subsequently dried, the transesterification activity was restored (17th run). Yields greater than 90% were obtained. Washing with a solution of $NaHCO_3$ removed the hydrophilic residues off the lipase, thereby restoring its transesterification activity.

Effect of Solvent. The ability of hydrophobic solvents to sustain and enhance enzyme catalysis has been repeatedly demonstrated (Hirata et al., 1990; Klibanov, 1989; Laane et al., 1987; Narayan and Klibanov, 1993; Welsh et al., 1989; Zaks and Klibanov, 1988). From a mechanistic standpoint, the effect of organic solvents on enzymatic catalysis is still debated (Hirata et al., 1990; Laane et al., 1987; Narayan and Klibanov, 1993). Solvents with high log *P* values are supposed to allow greater enzyme stability and generate high yields (Laane et al., 1987). However, inconsistencies have been reported, especially for substrates having chain lengths $\leq C_4$ (Narayan and Klibanov, 1993; Welsh et al., 1989). The effect of solvents having log *P* values ranging from −0.33 to 4.51 was investigated. Solvents having log *P* values ≥0.85 gave yields ≥61.7 and 75.6% for geranyl acetate and citronellyl acetate, respectively (Table 1). Despite a log *P* value of 0.71, pyridine totally suppressed the lipase activity. The lipase also performed very well in solvent-free synthesis of terpene esters. Although the geraniol samples that gave the lowest yields had the highest water contents, no direct relation was observed between the water content of the sample and the lipase activity.

We demonstrated that under the conditions used in this study *C. antarctica* lipase, SP435, was able to synthesize primary terpenyl acetates by transesterification reaction. These findings underscore the need to discover lipases that can exhibit a high affinity for short-chain fatty acids which are used in the synthesis of short-chain terpene esters that are important in the flavor industry (Gillis, 1988).

Table 1. Effect of Selected Organic Solvents on SP435 Lipase-Catalyzed Transesterification of Geranyl and Citronellyl Acetate

		geranyl acetate		citronellyl acetate	
solvent	log *P* value[a]	water content[b]	yield[c]	water content	yield
no solvent		nd[d]	94.1	nd	94.9
petroleum ether		243	95.9	87	96.1
isooctane	4.51	160	86.6	91	95.4
hexane	3.50	182	96.8	87	96.6
toluene	2.50	582	66.3	234	85.9
diethyl ether	0.85	1600	61.7	992	75.6
pyridine	0.71	2295	0.0	2410	3.1
tetrahydrofuran	0.49	974	38.3	436	53.4
acetonitrile	−0.33	539	30.0	400	50.2

[a] Source: Laane et al. (1987). [b] In ppm. [c] In percent molar conversion. [d] nd, not determined.

ACKNOWLEDGMENT

This work was contributed by the Agricultural Experiment Station, College of Agricultural and Environmental Sciences, The University of Georgia. Research was supported by Food Science Research 2526 GC 294000 and USDA Hatch Project GEO 00695.

LITERATURE CITED

Akoh, C. C. Lipase-catalyzed synthesis of partial glycerides. *Biotechnol. Lett.* **1993,** *15*, 949−954.

Akoh, C. C.; Cooper, C.; Nwosu, C. V. Lipase G-catalyzed synthesis of monoglycerides in organic solvent and analysis by HPLC. *J. Am. Oil Chem. Soc.* **1992,** *69*, 257−260.

Bloomer, S.; Aldercreutz, P.; Mattiasson, B. Triglyceride interesterification by lipases. 1. Cocoa butter equivalents from a fraction of palm oil. *J. Am. Oil Chem. Soc.* **1990,** *67*, 519−524.

Chopineau, J.; McCafferty, C. C. Synthesis of alkyl glycoside fatty acid esters in non-aqueous media by *Candida sp.* lipase. *J. Am. Oil Chem. Soc.* **1993,** *70*, 43−46.

Chulalaksananukul, W.; Condoret, J. S.; Combes, D. Kinetics of geranyl acetate synthesis by lipase-catalyzed transesterification in n-hexane. *Enzyme Microb. Technol.* **1992,** *14*, 293−298.

Chulalaksananukul, W.; Condoret, J. S.; Combes, D. Geranyl acetate synthesis by lipase-catalyzed transesterification in supercritical carbon dioxide. *Enzyme Microb. Technol.* **1993,** *15*, 691−698.

Claon, P. A.; Akoh, C. C. Enzymatic synthesis of geraniol and citronellol esters by direct esterification in n-hexane. *Biotechnol. Lett.* **1993,** *15*, 1211−1216.

Claon, P. A.; Akoh, C. C. Effect of reaction parameters on SP435 lipase-catalyzed synthesis of citronellyl acetate in organic solvent. *Enzyme Microb. Technol.* **1994,** in press.

Croteau, R. The biosynthesis of terpene compounds. In *Fragrance and Flavor Substances*; Croteau, R., Ed.; D & PS Verlag: Pattensen, Germany, 1980; pp 13−14.

de Castro, H. F.; Anderson, W. A.; Moo-Young, M.; Legge, R. L. Partitioning of water during the production of terpene esters using immobilized lipase. In *Biocatalysis in Non-Conventional Media;* Tramper, J., Vermüe, M. H., Beeftink, H. H., von Stockar, U., Eds.; Elsevier Science Publishers: New York, 1992; Vol. 8, pp 475−482.

Gillis, A. Research discovers new roles for lipases. *J. Am. Oil Chem. Soc.* **1988,** 65, 846−850.

Gray, C. J.; Narang, J. S.; Barker, S. A. Immobilization of lipase from *Candida cylindraceae* and its use in the synthesis of menthol esters by transesterification. *Enzyme Microb. Technol.* **1990,** *12*, 800−807.

Hirata, H.; Higuchi, K.; Yamashina, T. Lipase-catalyzed transesterification in organic solvent: effects of water and solvent, thermal stability and some applications. *J. Biotechnol.* **1990,** *14*, 57−167.

Iwai, M.; Okumura, S.; Tsujisaka, Y. Synthesis of terpene alcohol esters by lipase. *Agric. Biol. Chem.* **1980,** *44,* 2731–2732.

Kanasawud, P.; Phutrakul, S.; Bloomer, S.; Aldercreutz, P.; Mattiasson, B. Triglyceride interesterification by lipases. 3. Alcoholysis of pure triglycerides. *Enzyme Microb. Technol.* **1992,** *14,* 959–965.

Klibanov, A. M. Enzymes that work in organic solvents. *CHEMTECH* **1986,** *16,* 354–359.

Klibanov, A. M. Enzymatic catalysis in anhydrous organic solvents. *Trends Biochem. Sci.* **1989,** *14,* 141–149.

Laane, C.; Boeren, S.; Vos, K.; Cees, V. Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.* **1987,** *30,* 81–87.

Langrand, G.; Triantaphylides, C.; Baratti, J. Lipase catalyzed formation of flavour esters. *Biotechnol. Lett.* **1988,** *10,* 549–554.

Langrand, G.; Rondot, N.; Triantaphylides, C.; Baratti, J. Short chain flavor esters synthesis by microbial lipases. *Biotechnol. Lett.* **1990,** *12,* 581–586.

Macrae, A. R. J. Lipase-catalyzed interesterification of oils and fats. *J. Am. Oil Chem. Soc.* **1983,** *60,* 243A–246A.

Marlot, C.; Langrand, G.; Triantaphylides, C.; Baratti, J. Ester synthesis in organic solvent catalyzed by lipases immobilized on hydrophylic supports. *Biotechnol. Lett.* **1985,** *9,* 647–650.

Mukherjee, K. D. Lipase-catalyzed reactions for modification of fats and other lipids. *Biocatalysis* **1990,** *3,* 277–293.

Mutua, L. N.; Akoh, C. C. Synthesis of alkyl glycoside fatty acid esters in non-aqueous media by *Candida sp.* lipase. *J. Am. Oil Chem. Soc.* **1993,** *70,* 43–46.

Narayan, V. S.; Klibanov, A. M. Are water-immiscibility and apolarity of the solvent relevant to enzyme efficiency? *Biotechnol. Bioeng.* **1993,** *41,* 390–393.

Novo Industri. *Novo's Handbook of Practical Biotechnology,* 2nd ed.; Boyce, C. O. L., Ed.; Bagsvaerd, Denmark, 1986; pp 48–50.

Novo Industri. Product Information, B 347c-GB, Dec 1992.

Okumura, S.; Iwai, M.; Tjujisaka, Y. Synthesis of various kinds of esters by four microbial lipases. *Biochim. Biophys. Acta* **1979,** *575,* 156–165.

Welsh, W. F.; Murray, W. D.; William, R. E. Microbiological and enzymatic production of flavor and fragrance chemicals. *Crit. Rev. Biotechnol.* **1989,** *9,* 105–108.

Yamane, T. Enzyme technology for lipid industry: an engineering overview. *J. Am. Oil Chem. Soc.* **1987,** *64,* 1657–1662.

Zaks, A.; Klibanov, A. M. The effect of water on the enzyme action in organic media. *J. Biol. Chem.* **1988,** *263,* 8017–8021.

Received for review March 2, 1994. Revised manuscript received July 25, 1994. Accepted July 29, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1994.