Lipase PS-Catalyzed Transesterification of Citronellyl Butyrate and Geranyl Caproate: Effect of Reaction Parameters

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ABSTRACT: *Pseudomonas* sp. lipase PS was immobilized by adsorption and tested for its ability to catalyze the synthesis of citronellyl butyrate and geranyl caproate by transesterification in *n*-hexane. The reaction parameters investigated were: enzyme load, effect of substrate concentration, added water, temperature, time course, organic solvent, pH memory, and enzyme reuse. Yields as high as 96 and 99% were obtained for citronellyl butyrate and geranyl caproate, respectively, with 300 units (approx. 15% w/w of reactants) of lipase PS. Increasing amounts of terpene alcohol inhibited lipase activity, while excess acyl donor (triacylglycerol) concentration enhanced ester production. Optimal yields were obtained at temperatures from 30–50°C after 24-h incubation time. Yields of 90 and 99% were obtained for citronellyl and geranyl esters, respectively, with 2% added water. Solvents with log *P* values ≥2.5 showed the highest conversion yields. pH 7 and 6–8 seemed to be ideal for citronellyl butyrate and geraniol caproate, respectively. The lipase remained active after reusing 12 times.
JAOCS 74, 255–260 (1997).

KEY WORDS: Adsorption, citronellyl butyrate, geranyl caproate, immobilization, lipase, *Pseudomonas* sp., reaction parameters, transesterification.

Esters of terpene alcohols, such as citronellol and geraniol, are important flavor and fragrance compounds. They occur naturally in many essential oils, and, because of their organoleptic properties, are used to give flowery-fruity notes in a variety of products, such as foods, detergents, soaps, and perfumes. With consumer interest and preference for natural products, increasing attention has been focused on the use of enzymes to catalyze the synthesis of terpene esters. Lipases (triacylglycerol acylhydrolase E.C. 3.1.1.3) can be used to obtain flavor esters through direct esterification (1–7) and transesterification reactions (8–14). However, fewer studies have focused on the production of terpene esters by the latter method. In our previous study, lipase PS was immobilized by adsorption onto five different supports and tested for its ability to catalyze the synthesis of geranyl esters by transesterification of triacylglycerols of different chainlengths (14). The current paper focuses on the reaction parameters that affect immobilized *Pseudomonas* sp. lipase (PS)-catalyzed transesterification of citronellyl butyrate and geranyl caproate by using tributyrin and tricaproin, respectively, as acyl donors. This enzyme gave the highest yields of citronellyl butyrate and geranyl caproate of all acyl donor chainlengths studied. For industrial production and application, it is important that the best reaction conditions or parameters be determined.

MATERIALS AND METHODS

Materials. Nonspecific lipase PS (33,400 U/g) from *Pseudomonas* sp. in powdered form was obtained from Amano International Enzyme Co. (Troy, VA). DL-Citronellol (95% pure), geraniol (95% pure), tributyrin and tricaproin (99% pure), and support matrix polyvinylpyrolidone (PVP) were obtained from Sigma Chemical Co. (St. Louis, MO). *n*-Hexane and all other solvents (high-performance liquid chromatography grade) were purchased from Fisher Scientific (Norcross, GA). Duolite A-340 ion-exchange resin was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Immobilization procedure. Lipase PS was immobilized by a simple adsorption technique onto PVP and Duolite to catalyze the synthesis of citronellyl butyrate and geranyl caproate, respectively. PVP and Duolite were chosen as the immobilization matrices based on our previous report (14). Briefly, 1 g of support was washed three times with 5 mL distilled water and dried in an oven at 80°C. Powdered lipase PS (1 g) was dissolved in 1 mL distilled water. The dried support was added to the enzyme solution, mixed well with a stirring rod, and spread onto filter paper. This was then dried in a desiccator overnight at room temperature and stored in glass vials in the refrigerator until further use.

Transesterification method. Ester synthesis was carried out in duplicate as previously described by Yee *et al.* (13) in which 0.1 moles terpene alcohol and 0.03 moles triacylglycerol were added to 2 mL hexane, followed by 200 units (based

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on the mass of enzyme adsorbed) of immobilized lipase PS in screw-capped test tubes. Samples were incubated in an orbital waterbath at 30°C, 200 rpm for 24 h, along with their controls (samples with no enzymes). For solvent-free samples, reaction products were dissolved in 2 mL hexane after incubation and prior to analysis.

To study the effect of pH on terpene ester synthesis, the powdered lipase was dissolved in buffer solutions of different pH (0.1 M sodium citrate, sodium biphosphate and glycine, and adjusted to various pH values by using concentrated HCl or NaOH). The buffer preparations were used to immobilize lipase PS, then lyophilized and stored as described above.

Extraction and analysis. After 24 h of incubation, samples were removed, cooled in ice, and passed through an anhydrous sodium sulfate (Na_2SO_4) column to remove the enzyme and any residual water. Internal standard 200 μg (± linalool) was added to each sample. Product analysis was performed by injecting a 1-μL aliquot in a splitless mode into a gas–liquid chromatograph (GLC) (Hewlett-Packard HP 5890 Series II; Hewlett-Packard, Avondale, PA), equipped with a flame-ionization detector (FID). A DB-5 fused-silica capillary column (30 m × 0.32 mm i.d.; J & W Scientific, Folsom, CA) was used. Injector and detector temperatures were set at 250 and 260°C, respectively. For citronellyl butyrate, the temperature gradient was 150 to 200°C at 40°C/min. For geranyl caproate, the oven temperature was 150 to 210°C at 50°C/min. Helium was used as the carrier gas, and total flow rate was 24 mL/min. The synthesis of terpene esters was determined from the amount of terpene alcohol consumed and was further quantified by an on-line computer.

For enzyme reuse studies, after the incubation, reaction products were removed and passed through an anhydrous sodium sulfate column (12). The enzyme remaining in the test tube was rinsed with hexane and vortexed. The solvent was evaporated, and the lipase was dried and stored in a desiccator until the next use.

RESULTS AND DISCUSSION

Enzyme load. The amount of enzyme used in a reaction is of economic importance to any process. Figure 1 shows the effect of immobilized enzyme load on the synthesis of citronellyl butyrate and geranyl caproate. As the amount of enzyme was increased from 100 to 300 units, the product yields increased, then leveled off up to 400 units of lipase. So, 300 units (which corresponds to approximately 15% w/w of the reactants) seemed to be the optimal amount of enzyme, which resulted in yields of 98.6 and 94.2% for citronellyl butyrate and geranyl caproate, respectively.

Effect of substrate concentration. The effects of terpene alcohol and acyl donor concentration on the transesterification reaction catalyzed by lipase PS were investigated. As shown in Figure 2A, increasing the amounts of citronellol and geraniol had inhibitory effects on lipase PS activity. For both esters, the largest drop was seen at 0.4 M terpene alcohol concentration for which as much as a 35 and 42% decrease in

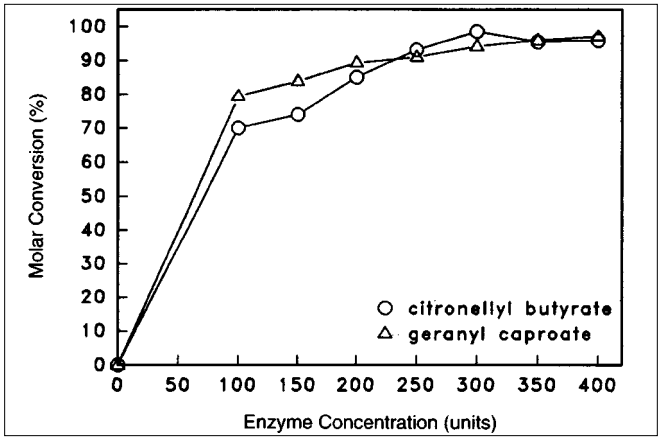

FIG. 1. Effect of immobilized enzyme load on synthesis of citronellyl butyrate and geranyl caproate. Samples were prepared by adding 0.1 moles terpene alcohol, 0.03 moles triacylglycerol to 2 mL *n*-hexane and incubating for 24 h with 0–400 units of *Pseudomonas* sp. lipase.

yields were obtained for citronellyl and geranyl esters, respectively. Chulalaksananukul *et al.* (10) reported the inhibitory effect of geraniol on the activity of *Mucor miehei* lipase, IM 20. Claon and Akoh (11) observed the same with two *M. miehei* lipases but not for *Candida antarctica* lipases. Welsh and Williams (15) noticed that the concentration of substrate used could affect enzyme performance, but the effect was dependent on the enzyme used to catalyze the reaction.

The effect of acyl donor concentration is also presented (Fig. 2B). For both esters, increasing the amounts of triacylglycerols did not inhibit lipase PS activity.

Effect of added water. The importance of water in biocatalytic reactions cannot be overemphasized. Water is required by most enzymes to maintain their catalytic activities (16). This required water, although minimal, is referred to as the "essential" or "biological water," and the enzyme needs it to keep its three-dimensional structure (16). Therefore, if water is removed, the protein's conformation is drastically distorted, and the enzyme is inactivated (17). However, it is uncertain what the nature and extent of this adsorbed water layer are and what interactions with the protein are important to sustain catalysis (18).

Figure 3 shows the effect of added water on terpene ester synthesis. The highest conversion yields were obtained when 2% water was added (90.0 and 98.9% for citronellyl and geranyl esters, respectively). At 30% added water, a 24.0 and 28.0% drop in yield for citronellyl and geranyl esters was observed. In the case of geranyl caproate, more than 10% water seemed to have an increasing negative effect on lipase PS activity. Excess water leads to competition between hydrolysis and transesterification. It was also observed that increasing amounts of water had a greater effect on geranyl caproate samples than on citronellyl butyrate. This is probably due to the fact that the geraniol had a relatively higher original water content than citronellol (12).

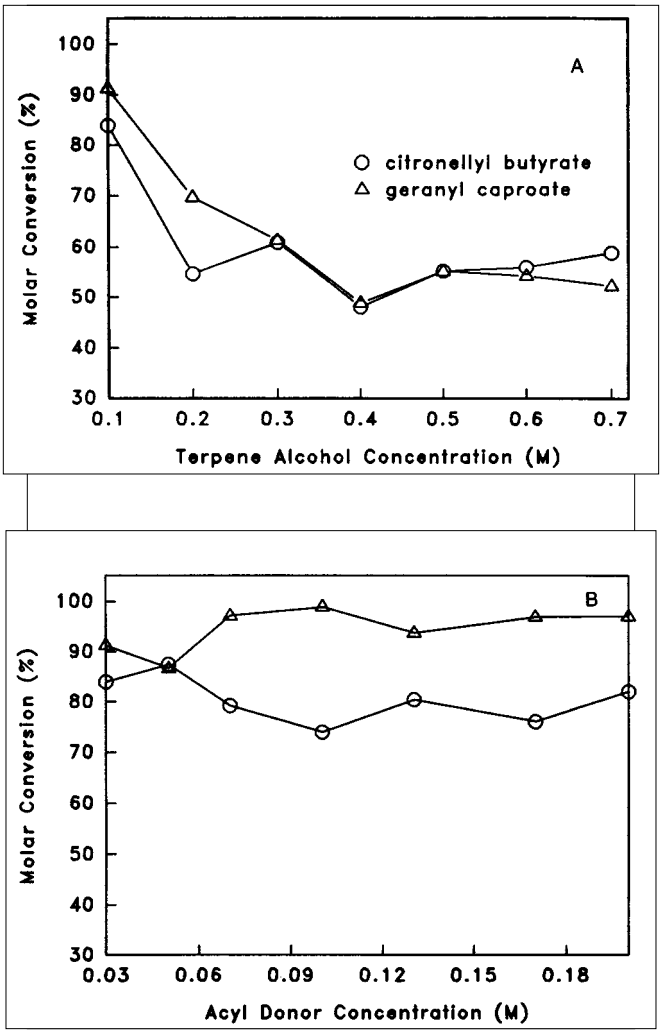

FIG. 2. Effect of substrate concentration on the synthesis of citronellyl butyrate and geranyl caproate over a 24-h period. Terpene alcohol effect (A): 0.03 M triacylglycerol was added to 2 mL *n*-hexane containing 0.1–0.7 M citronellol and geraniol, respectively and 200 units of lipase PS. Acyl donor effect (B): 0.1 M citronellol and geraniol were used with increasing concentrations of tributyrin and tricaproin, respectively.

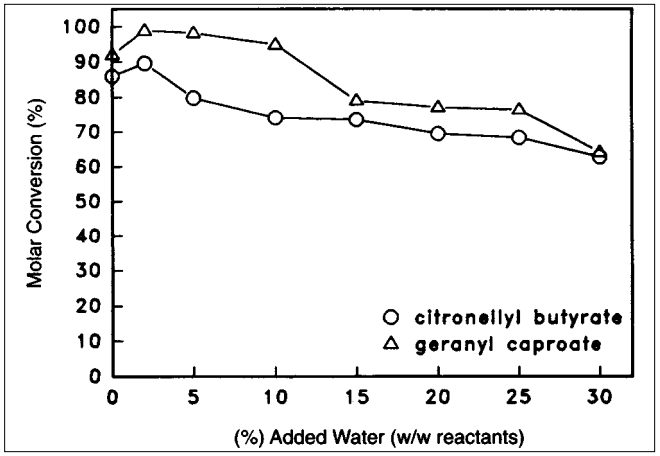

FIG. 3. Effect of added water on lipase PS-catalyzed synthesis of citronellyl butyrate and geranyl caproate. Increasing amounts of water, 0–30% (w/w of reactants), were added to reaction mixtures and incubated for 24 h.

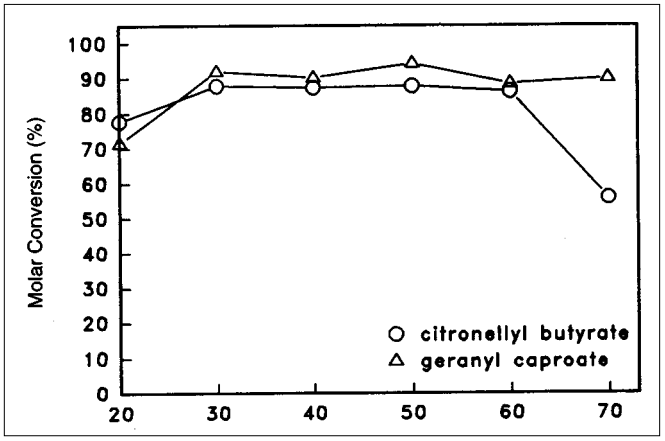

FIG. 4. Effect of temperature on lipase PS-catalyzed synthesis of citronellyl butyrate and geranyl caproate in *n*-hexane. Temperatures ranged from 20–70°C and reactions were for 24 h.

Effect of temperature. Figure 4 shows the effect of temperature on geranyl and citronellyl ester synthesis. Incubation temperatures ranged from 20 to 70°C, but optimal yields were obtained between 30–50°C for both esters. For geranyl caproate, good lipase activity was still maintained at 70°C. This is more than likely attributed to enzyme immobilization, which has the advantage of conferring stability to the *Pseudomonas* lipase. Also, geranial caproate is less volatile than the short-chain citronellyl butyrate at 70°C. An enzyme acquires greater resistance toward thermal inactivation after immobilization and can be used at higher temperatures to achieve faster reaction rates (19).

Time course. The time course is a good indicator of enzyme performance and reaction progress. It can pinpoint the shortest or adequate time necessary to obtain good yields and minimize process expenses. Yields of 60.7 and 75% for citronellyl butyrate and geranyl caproate, respectively, were observed within 1 h (Fig. 5). Yields for both esters increased with time, and highest yields were obtained after 24 h (88 and 93%, for citronellyl butyrate and geranyl caproate, respectively).

Effect of organic solvent. The use of organic solvents rather than aqueous media allows for synthetic reactions, decreases undesirable side reactions, enables product and enzyme recovery, increases thermostability, and eliminates microbial contamination (20,21). However, the use of organic solvents is limited because they can be potentially toxic, flammable, and may inactivate or denature the enzyme. Therefore, proper selection of organic solvent is required for specific syntheses to take place. The hydrophobicity of the organic

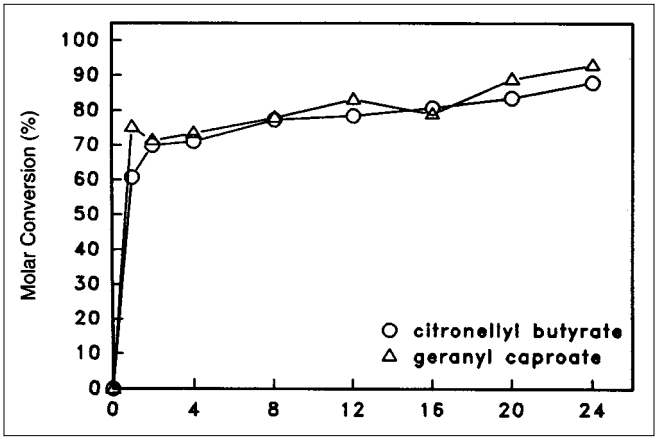

FIG. 5. Time course of enzymatic synthesis of citronellyl butyrate and geranyl caproate in *n*-hexane using *Pseudomonas* sp. lipase. Samples were analyzed after 1, 2, 4, 8, 12, 16, 20, and 24 h incubation.

TABLE 1
Effect of Organic Solvents on Lipase PS-Catalyzed Synthesis of Citronellyl Butyrate and Geranyl Caproate

Solvent[a]	log *P* value[b]	Molar conversion (%) Citronellyl butyrate	Molar conversion (%) Geranyl caproate
No solvent	—	97.0	93.1
Petroleum ether	—	93.1	83.9
Isooctane	—	90.2	95.2
n-Hexane	3.50	90.2	95.2
Cyclohexane	3.20	93.6	83.3
Pentane	3.00	96.1	92.9
Toluene	2.50	16.8	66.0
Tetrahydrofuran	0.49	—	53.7
Acetone	–0.23	61.2	55.2
Acetonitrile	–0.33	14.9	68.6

[a]Solvents were dried over molecular sieve 4 Å.
[b]Source: Reference 21.

solvent plays a major role in determining catalytic activity (16). The log *P* value, which is the partition coefficient between water and octanol, is a good indicator of solvent polarity. Therefore, proper solvents can be chosen based on their hydrophobicity. Generally, solvents with log $P \leq 2$ show little biocatalytic activity, while those with log $P \geq 4$ show high biocatalytic activity (16,21). It should be noted that in the absence of organic solvent, we observed comparable yields. Therefore, low water content or activity of the solvents and the reaction is equally important.

Table 1 shows the effect of various organic solvents on lipase PS transesterification activity. The highest yields were obtained with solvents having log *P* values >3. Specifically, iso-octane (90.2 and 95.2% yields for citronellyl and geranyl esters, respectively) and pentane (96.1 and 92.8%, respectively) gave the best results. Samples with no solvents also performed well (97 and 93.1% for citronellyl and geranyl, respectively), while solvents with log *P* values ≤2.5 exhibited the lowest yields. Polar solvents, due to their higher affinity to water, tend to strip the essential water from enzyme molecules (17) and cause inactivation.

Effect of pH memory. "pH memory" refers to the enzyme's ability to retain the catalytic characteristics of the pH of the last aqueous solution to which it was exposed (20). Ultimately, the activity and stability of an enzyme rely on the pH of the solution from which it is recovered. Because different enzymes have different pH optima, it is important to know the optimal pH for enzymatic activity. Furthermore, the optimal pH may differ for different substrates. Hence, a favorable pH range should be determined for a given reaction. This range depends not only on the nature of the enzyme, but also on the substrate, substrate concentration, stability of the enzyme, temperature, and length of the reaction period (19).

Figure 6 shows the effect of different pH values on the catalytic activity of lipase PS. For citronellyl butyrate, the optimal pH was 7 (88.7% yield). For geranyl caproate, pH 6 (94.4% yield) and 8 (97.3% yield) were the most suitable.

Effect of enzyme reuse. One of the main advantages of immobilized enzymes is that they can be reused, and consequently decrease process costs. Figure 7 illustrates the yields obtained for 12 runs. The results were rather erratic. For example, after the 5th run, a 39 and 28% drop in yields for citronellyl and geranyl esters, respectively, were observed. After the 10th run, the samples were washed with 10% $NaHCO_3$ solution to remove the hydrophilic residues from the lipase (12) and restore the enzyme transesterification activity. An initial increase in conversion yields was seen for the 11th run for both esters, but it decreased again for the 12th run. Claon and Akoh (12) also reported erratic behavior for enzyme reuse studies, in which they used a commercially available enzyme, SP 435, a *C. antarctica* lipase produced from a gene cloned into *Aspergillus oryzae* and immobilized onto polyacrylic resin. However, when 10% $NaHCO_3$ was used for the same purpose as in this experiment, the transesterification ac-

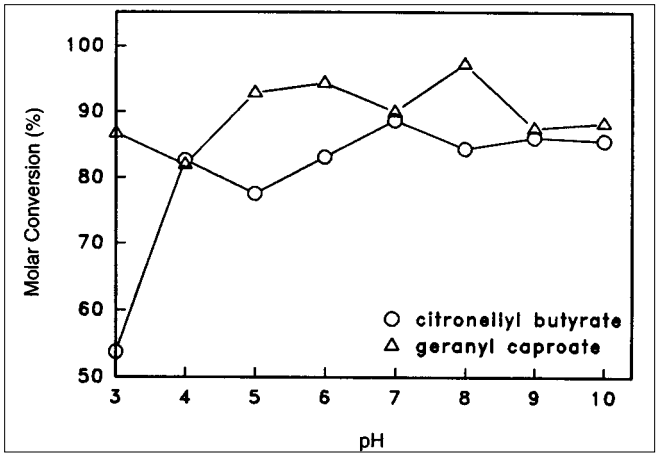

FIG. 6. Effect of pH memory on immobilized lipase PS-catalyzed synthesis of citronellyl butyrate and geranyl caproate in *n*-hexane for 24 h. The enzyme was dissolved in buffer solutions of pH 3–10. The preparations were then immobilized and lyophilized.

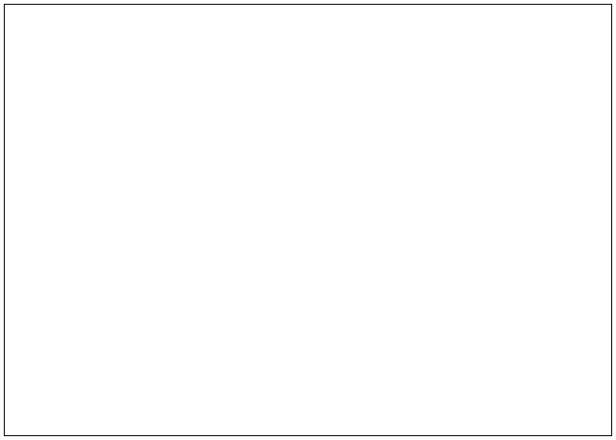

FIG. 7. Effect of reuse of immobilized lipase PS on synthesis of citronellyl butyrate and geranyl caproate by transesterification in *n*-hexane. After each 24-h run, the enzyme was washed with hexane and the solvent evaporated prior to reuse.

tivity was greatly restored (12). Our results here were not as successful. We speculate that this is probably due to the immobilization technique used. Adsorption methods, although simple and easy to perform, rely on physical and ionic interactions, and enzymes can easily be desorbed from the support. In nonaqueous media, enzymes are not normally soluble in organic solvents. Thus, simple techniques, such as adsorption, can be used (22) provided that synthetic reactions occur and good conversion yields are obtained. Despite this disadvantage, adsorption techniques have been used for some industrial processes (23) and have been shown to work successfully in transesterification and esterification reactions in nonaqueous systems (24), in particular, in the production of short-chain flavor esters (25).

Under the conditions of this study, we have demonstrated that lipase PS can synthesize citronellyl butyrate and geranyl caproate by transesterification reactions. By testing the parameters that affect lipase PS activity on terpene ester synthesis, we have shown that suitable conditions can be selected to optimize its biocatalytic activity, as well as promote their use for the production of commercial flavor esters.

ACKNOWLEDGMENTS

This work was supported by the Agricultural Experimental Station, College of Agricultural and Environmental Sciences and The University of Georgia Research Foundation Biotechnology Grant.

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[Received June 10, 1996; accepted January 28, 1997]