Enzymatic Synthesis of Geranyl Acetate by Transesterification with Acetic Anhydride as Acyl Donor

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ABSTRACT: *Pseudomonas* sp. lipase (PS) was immobilized by adsorption technique onto glass beads and tested for its ability to synthesize geranyl acetate by transesterification with acetic anhydride as the acyl donor. Reactions were carried out in *n*-hexane containing 0.1 M geraniol, 0.1 M acetic anhydride, and 200 units of lipase PS. Enzyme load, effect of substrate concentration, added water, temperature, time course, organic solvent, pH memory, and enzyme reuse were studied. Yields of up to 96% were obtained with 200 units (approximately 11% w/w of reactants) of enzyme. Increasing amounts of geraniol inhibited lipase activity, while excess acyl donor concentration enhanced ester production. Yields as high as 97% were obtained at 50°C, 24 h incubation, with no added water. Solvents with log *P* values ≥3.0 showed the highest conversion yields. Solvent-free samples also performed well. The pH range of 4–9 gave good yields (92–98.4%). Enzyme reuse studies showed the lipase remained active after 15 runs.
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KEY WORDS: Acetic anhydride, adsorption, geranyl acetate, immobilization, lipase, *Pseudomonas* sp., reaction parameters, terpene esters, transesterification.

Esters of terpene alcohols, such as citronellol and geraniol, especially the acetates, are found in many essential oils and are commonly used as flavor and fragrance compounds. As a flavor, geranyl acetate is mainly used to create fruity aromas in a variety of foods and beverages (1). Mass production of terpene esters is usually done by chemical synthesis. However, as a response to consumer demand for natural products, coupled with the realization that enzymes, particularly lipases, are active and stable in organic solvents (2), a lot of attention has been focused on the application of lipases to catalyze various reactions. These include synthesis of partial glycerols for use as emulsifers (3–5), production of alkyl glycoside fatty esters for use as biosurfactants (6), modification of fats and oils to improve their physical and nutritional properties (7), and biocatalysis for the production of flavor esters (8–11).

Most studies, dealing with enzymatic synthesis of terpenyl acetates by direct esterification, have reported low yields due to the inhibitory effect of acetic acid on lipase activity (12–14). Few studies have succeeded in improving their yields (8,9,15). To circumvent or reduce this effect, lipase-catalyzed transesterification has also been reported with propyl acetate (16), novel acyl donors, such as methyl, ethyl, isopropenyl, and isoamyl acetates (17), and triacetin (17,18). Fatty acid anhydrides are being used as novel acyl donors. Shaw and Lo (4) reported their use as best substrates for the *Pseudomonas* sp. lipase (PS)-catalyzed synthesis of propylene glycol monoesters.

In this study, acetic anhydride was used as acyl donor for the enzymatic synthesis of geranyl acetate by PS. Enzyme load, effect of substrate concentration, added water, temperature, time course, organic solvent, pH memory, and enzyme reuse were investigated.

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). Geraniol (95% pure) was obtained from Sigma Chemical Co. (St. Louis, MO). Acetic anhydride was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Glass beads (3 mm) were obtained from Corning Incorporated (Corning, NY). *n*-Hexane and all other solvents (high-performance liquid chromatography-grade) were purchased from Fisher Scientific (Norcross, GA).

Immobilization procedure. Lipase PS was immobilized by simple adsorption technique onto glass beads, as described by Yee *et al.* (11). Briefly, 1 g of support was washed three times with 5 mL distilled water and dried in an oven at 80°C, and 1 g of powdered lipase PS 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 use.

Transesterification method. Ester synthesis was carried out in screw-capped test tubes in duplicates as previously described by Yee *et al.* (10) in which 0.1 M geraniol and 0.1 M acetic anhydride were added to 2 mL hexane, followed by 200 units of immobilized lipase PS. Samples were incubated in an orbital water bath at 30°C and 200 rpm for 24 h, along with their controls (samples with no enzymes).

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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 geranyl acetate 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 incubation, samples were removed and washed with 1 mL 10% $NaHCO_3$ to neutralize the acid; 100 µg of internal standard (ethyl caproate) was added to each sample before being passed through an anhydrous sodium sulfate (Na_2SO_4) column to remove the enzyme and any residual water. Product analysis was performed by injecting a 0.8-µL aliquot in a splitless mode into a gas–liquid chromatograph (Hewlett-Packard HP 5890 Series II; Hewlett-Packard, Avondale, PA), equipped with a flame-ionization detector. 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. The temperature gradient was 150 to 190°C, at 10°C/min. Helium was the carrier gas, and total flow was 24 mL/min. Synthesis of geranyl acetate was determined from the amount of geraniol consumed and was further quantitated by an on-line computer.

For enzyme reuse studies, after the incubation, reaction products were removed and passed through an anhydrous sodium sulfate column (18). 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

With enzymes costing as much as $1500 U.S./kg (19), the amount of enzyme used is crucial for enzymatic processes to be competitive with chemical processes. The effect of enzyme load is shown in Figure 1. As the amount of enzyme increased from 0 to 400 units, yields of geranyl acetate increased accordingly; 200 units (which corresponds to approximately 11% w/w of the reactants) gave a yield of up to 96%. Only a 2% increase was obtained with 400 units (approximately 23% w/w of reactants).

The effect of geraniol and acetic anhydride concentrations on lipase PS transesterification activity was investigated. Increasing amounts of geraniol concentration inhibited lipase PS activity (Fig. 2A). A 43% drop in yield was observed at 0.3 M geraniol. At 0.7 M, yield dropped to 24%, whereas for control samples, product formation was almost null at such high concentrations. Obviously, the acyl donor was consumed by the high concentration of geraniol. This trend was observed by other investigators. Chulalaksananukul *et al.* (16) reported the inhibitory effect of geraniol on *Mucor miehei* lipase (IM 20) activity. Claon and Akoh (8) reported the same effect with two *M. miehei* lipases (IM 20 and IM 60), but not for *Candida antarctica* lipases (SP 382 and SP 435). It seems

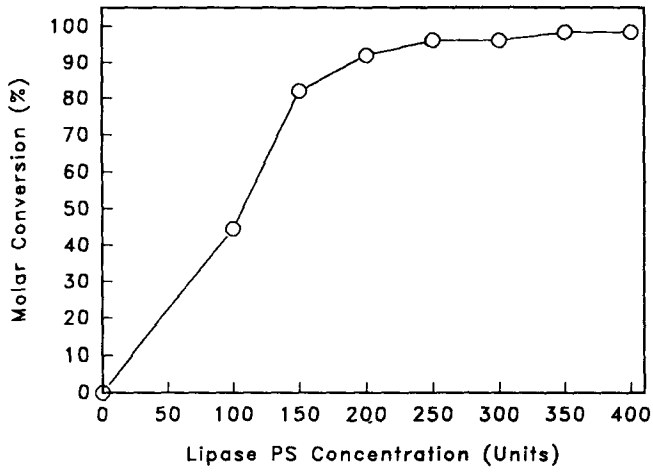

FIG. 1. Effect of enzyme load on synthesis of geranyl acetate. Samples were prepared by adding 0.1 M geraniol and 0.1 M acetic anhydride to 2 mL *n*-hexane and incubating with 0–400 units of *Pseudomonas* sp. lipase (PS).

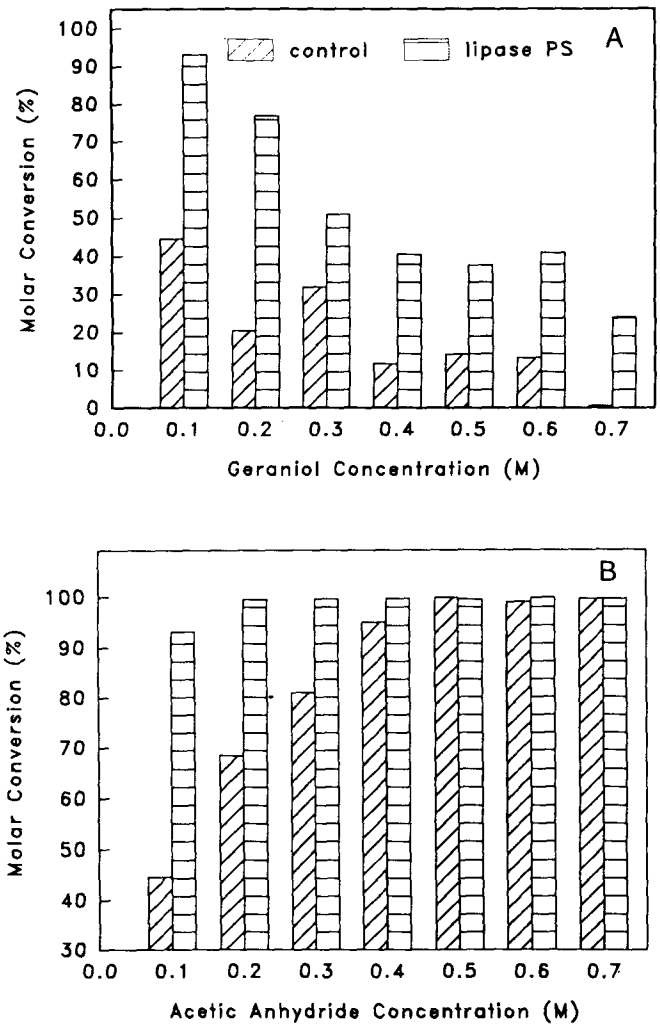

FIG. 2. Effect of substrate concentration on the transesterification activity of lipase PS. Terpene alcohol effect (A): 0.1 M acetic anhydride was added to 2 mL *n*-hexane containing 0.1–0.7 M geraniol and 200 units of lipase PS. Acyl donor effect (B): 0.1 M geraniol was used with increasing concentrations of acetic anhydride. See Figure 1 for abbreviation.

that terpene alcohols inhibit lipase performance, but the degree of inhibition depends on the enzyme used (20). However, increasing amounts of acetic anhydride did not seem to inhibit lipase PS activity (Fig. 2B). For all samples (including controls), yields improved as acyl donor concentration increased. At 0.2 M acetic anhydride, a 99.5% yield was observed and remained constant up to 0.7 M concentration. For controls, at 0.5 M a 99.8% conversion yield was obtained. Thus, excess amounts of acid anhydride increased ester formation, even without enzyme.

It is well established that, for almost all enzymes, a layer of adsorbed water is essential to promote catalytic activity in organic solvents (21,22). However, critical water contents for optimal activity vary widely as other parameters, such as enzyme, support, and solvent used, are changed (23). The optimum amount of water should be a compromise between minimizing hydrolysis and maximizing enzyme activity for synthesis (16,24). The effect of added water is shown in Figure 3. Lipase PS performed best under anhydrous conditions (92% yield). As the amount of added water increased, competition between hydrolysis and transesterification became more obvious. At 20% added water, a 67% drop in yield was observed. However, after 25 and 30% added water, a partial restoration of lipase PS activity was observed. The exact reason for this phenomenon is unclear, but restoration of esterification activity after a steady drop has been reported in other studies with *C. antarctica* lipases (6,15).

Synthesis of geranyl acetate improved as the incubation temperature increased (Fig. 4). Best yields were obtained between 30–60°C, with a 97% yield at 50°C. Lipase PS was shown to have good catalytic activity over a wide temperature range, due in part to the stability conferred by immobilizing the enzyme (10,25).

Time course is a good indicator of enzyme performance and reaction progress. It helps determine the shortest time necessary to obtain good yields, thereby making the process cost-effective. Figure 5 shows the time course for lipase PS-catalyzed synthesis of geranyl acetate. After 24 and 48 h, yields of 92 and 97%, respectively, were observed. Only a 5% increase was obtained after 24 h. Interesting, controls with no enzymes gave a 44.6% yield after 24 h (data not shown). Thus, acetic anhydride was highly reactive, even without enzyme.

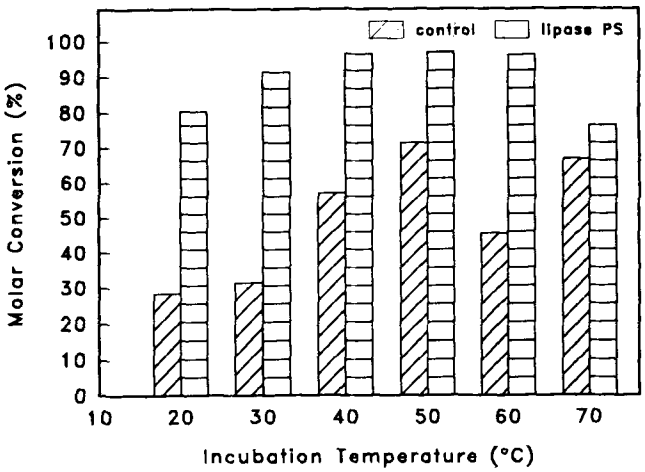

FIG. 4. Effect of temperature on *Pseudomonas* sp. lipase-catalyzed synthesis of geranyl acetate in *n*-hexane.

Use of organic solvents affects the performance of enzymes, resulting in altered stability, specificity, and enantioselectivity (26). The use of organic solvents is limited, however, because they can denature or inactivate enzymes. Thus, proper selection of organic solvents is required. However, immobilization often imparts a markedly enhanced stability against denaturation. Efficient interactions between the enzyme and appropriate support allow the enzyme to maintain its active conformation, which prevents inactivation by organic solvents (27). It has been shown that solvent hydrophobicity influences enzymatic activity (22). A good indicator of solvent polarity is the log *P* value, which is the partition coefficient between water and octanol (28). Therefore, proper sol-

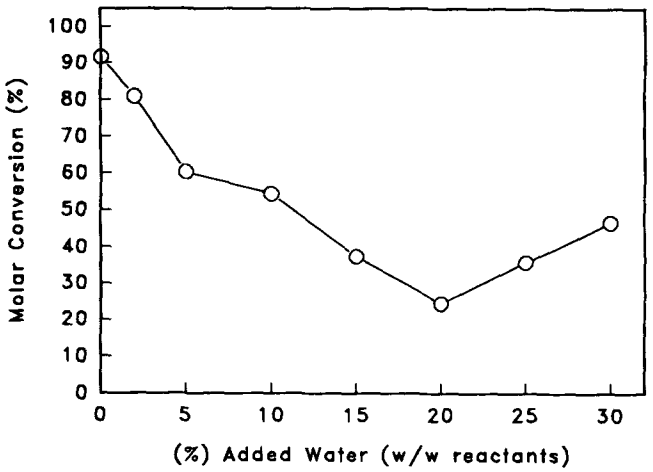

FIG. 3. Effect of added water on *Pseudomonas* sp. lipase-catalyzed synthesis of geranyl acetate. Increasing amounts of water, 0–30% (w/w of reactants), were added to reaction mixtures.

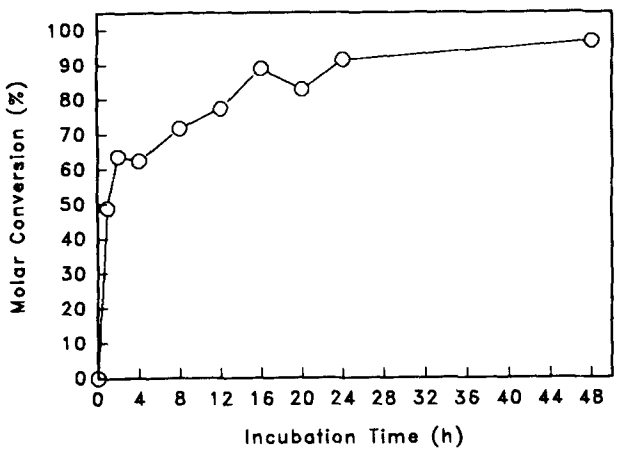

FIG. 5. Time course of enzymatic synthesis of geranyl acetate in *n*-hexane with *Pseudomonas* sp. lipase. Samples were analyzed after 1, 2, 4, 8, 12, 16, 20, 24, and 48 h of incubation.

vents can be selected based on their hydrophobicity. Generally, solvents with log $P \leq 2$ allow little biocatalytic activity, while those with log $P \geq 4$ allow high biocatalytic activity (22,28). Table 1 presents the effect of various organic solvents on lipase PS-catalyzed transesterification of geranyl acetate. Highest yields were obtained with solvents of log P values ≥ 3.0, with petroleum ether, hexane, and pentane giving yields of 95, 91.6, and 94.2%, respectively. Samples with no solvent also performed well (91.2%). In general, solvents with log P values ≤ 3.0 exhibited lower yields. Being more hydrophilic in nature, they tend to disturb the water layer found around enzyme molecules, which is essential to their activity (29).

pH memory refers to the enzyme's ability to remember the pH of the last aqueous solution it was exposed to (2). The activity and stability of an enzyme relies on the pH of the solution from which it is recovered or stored. It is important to know the optimal pH for enzymatic activity because different enzymes have different pH optima, which may differ for different substrates. 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 (30,31). The effect of pH memory on the catalytic activity of lipase PS is shown in Figure 6. Except for pH 3, the pH range between 4–9 gave good yields (92–98.4%). At present, the exact reason for this behavior is poorly understood. We also observed a decrease at pH 5 for which we have no explanation. It is known that lipase PS has lipolytic activity within a wide pH range of 3–11, and optimum pH is 7–8 (19). We recently studied the effect of pH memory on lipase PS-catalyzed synthesis of citroellyl butyrate and geranyl caproate (Yee, L.N., and C.C. Akoh, 1995, unpublished observation). Optimum pH ranges were found, but did not cover as wide a range as in this study.

Immobilization allows for expensive enzymes to be recovered and reused in a system, thereby decreasing process costs and permitting continuous automated production (32). Yields obtained after 15 runs are reported (Fig. 7). Loss of esterification activity was only 4% after the first two runs. However,

TABLE 1
Effect of Organic Solvents on *Pseudomonas* sp. Lipase-Catalyzed Synthesis of Geranyl Acetate

Solvent[a]	log P value[b]	Molar conversion (%)	
		Control	Lipase PS
No solvent	—	33.6	91.2
Petroleum ether	—	13.8	95.0
Iso-Octane	4.50	11.1	80.4
n-Hexane	3.50	20.4	91.6
Cyclohexane	3.20	29.8	73.4
Pentane	3.00	61.5	94.2
Toluene	2.50	11.9	75.6
Tetrahydrofuran	0.49	6.0	50.5
Acetone	−0.23	3.8	77.8
Acetonitrile	−0.33	—	69.1

[a]Solvents were dried over molecular sieve 4 Å.
[b]Source: Reference 28.

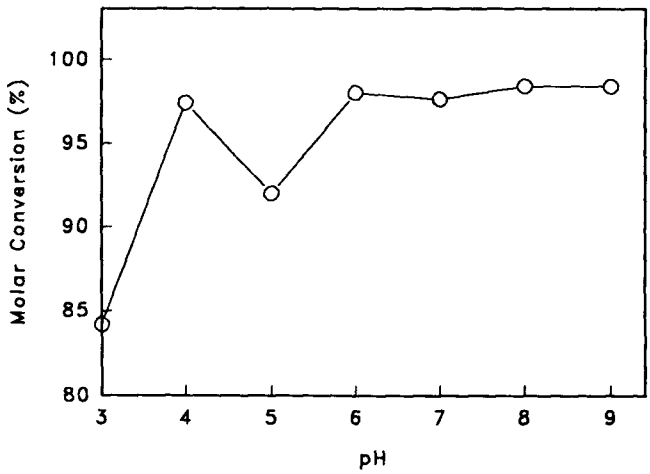

FIG. 6. Effect of pH memory on immobilized *Pseudomonas* sp. lipase-catalyzed synthesis of geranyl acetate in *n*-hexane. The enzyme was dissolved in buffer solutions of pH 3–9. The preparations were then immobilized and lyophilized as described in the text.

after the third run as much as a 28% drop in yield was observed. After that, results became rather erratic. After the eleventh run, samples were washed with $NaHCO_3$ to remove the hydrophilic residues from the lipase (18) and restore the enzyme transesterification activity. An initial increase in conversion yields was observed after the twelveth and thirteenth runs, but decreased dramatically for the fourteenth and fifteenth runs. This is not the first time that inconsistencies in lipase reuse are reported. Claon and Akoh (18) observed similar behavior with enzyme reuse studies on a commercially immobilized enzyme, SP 435, a *C. antarctica* lipase cloned into *Aspergillus oryzae* and immobilized onto polyacrylic resin. However, when $NaHCO_3$ was used for the same purpose as in this experiment, the transesterification activity was greatly restored. We previously encountered the same situation with citronellyl and geranyl esters, and speculated that it is attrib-

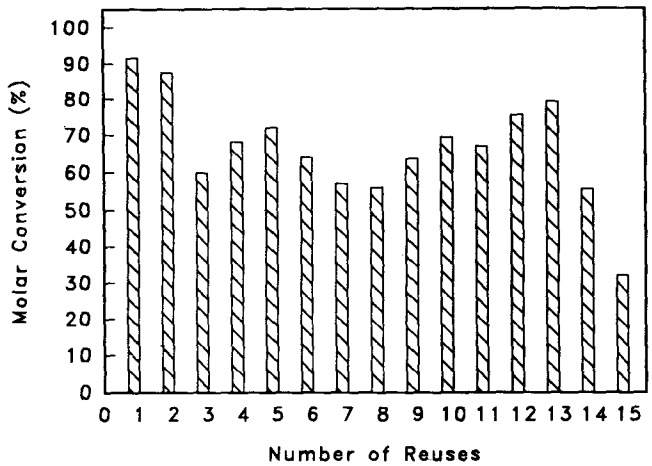

FIG. 7. Effect of immobilized *Pseudomonas* sp. lipase reuse on synthesis of geranyl acetate by transesterification in *n*-hexane. After each run, the enzyme was washed with hexane and solvent evaporated prior to reuse.

uted to the immobilization technique used. Adsorption methods have the advantage of being simple and easy to prepare, relying on physical and ionic interactions. The drawback is that 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 still be utilized (33), provided that synthetic reactions occur and good conversion yields are obtained. Despite this disadvantage, adsorption techniques have been used for some industrial processes (34) and shown to work successfully in transesterification and esterification reactions in nonaqueous systems (35), particularly in the production of short-chain flavor esters (36).

To the best of our knowledge, this work represents the first comprehensive study on the parameters that affect enzymatic synthesis of geranyl acetate by PS-catalyzed transesterification with acetic anhydride as the acyl donor. Numerous other studies have focused on terpene ester synthesis by lipases from *M. miehei* IM 20 (14,16,36–38); *C. cylindraecae* (39,40), and *C. antarctica* SP 435 (8,9,15,17). By investigating the parameters affecting geranyl acetate synthesis, we have pinpointed the necessary conditions for proper scale-up, with the hope that they can be further optimized for the enzymatic synthesis of commercial flavor esters.

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