Enzymatic Synthesis of Geranyl Acetate in *n*-Hexane with *Candida antarctica* Lipases

Paul A. Claon and **Casimir C. Akoh***
Department of Food Science and Technology, The University of Georgia, Athens, Georgia 30602-7610

Geranyl acetate is an important flavor and fragrance compound. Two immobilized *Candida antarctica* lipases, SP382 and SP435, were investigated for their use in the synthesis of geranyl acetate by direct esterification. Yields between 95 and 99% molar conversion were obtained with 2 and 15% (w/w reactants) of SP435 and SP382 lipases, respectively. Optimum yields were obtained at 0.1M acetic acid and 0.12M geraniol after 16-h incubation. No inhibitory effect was observed at increasing concentrations of geraniol. Addition of 60% (w/w reactants) water led to 50 and 60% reduction in the esterification activity of SP382 and SP435 lipases, respectively. The best yields were obtained at added water contents between 0–5% (w/w reactants). Solvents with a log *P* value of 0.85 or more gave reaction yields of more than 80% molar conversion. Higher log *P* values did not necessarily lead to higher conversion yields. The immobilized lipase SP382 was still active after reusing ten times in the direct esterification reaction.

KEYS WORDS: *Candida antarctica*, direct esterification, enzymatic synthesis, geranyl acetate, lipases, organic solvents.

Esters of geraniol, especially the acetate, are valuable flavor and fragrance compounds. Geranyl acetate is a major constituent of numerous essential oils, such as lime oil, which has considerable economic importance (1). Recent trends in consumer preference toward natural products indicates that biocatalysts may have an advantage over their chemical counterparts, and products of biocatalytic processes may be termed "natural" (2). In addition, biocatalysts have lower energy requirements than chemical catalysts and can be used to synthesize products of better quality (3). Lipases (triacylglycerol acylhydrolase EC 3.1.1.3) can be used as biocatalysts for the production of flavor esters by transesterification (3–7) and direct esterification reactions (8–15). The use of acetic acid as an acyl donor in transesterification and direct esterification reactions was previously attempted with relatively little or no success (4,5,8,9,12,16–19). Acetic acid has an inhibitory effect on most lipases. It lowers the pH of their microenvironment and interferes with their aqueous layer (4,9,13,17). Recently, Claon and Akoh (20) reported the synthesis of terpene esters of acetic acid by direct esterification with yields of more than 90% molar conversion. Here we report the effect of various reaction parameters (i.e., enzyme and substrate concentration, time course, temperature, water, solvents and enzyme reuse) on the direct esterification of geraniol with acetic acid by means of two immobilized lipases from *Candida antarctica*.

MATERIALS AND METHODS

Materials. Nonspecific lipases from *C. antarctica*, immobilized on acrylic resin, SP382 (40 BIU/g) and SP435 (7000 BLU/g; cloned into *Aspergillus oryzae*), were obtained from Novo Nordisk Bioindustrials, Inc. (Danbury, CT). Geraniol (95% pure) was purchased from Sigma Chemical Co. (St. Louis, MO). Acetic acid and all solvents were high-performance liquid chromatography-grade and obtained from Fisher Scientific (Norcross, GA). Molecular sieve 4Å was purchased from Davison Chemical (Baltimore, MD). The reagent for coulometric determination of water (Hydranal-Coulomat AG) was purchased from Crescent Chemical Co., Inc. (Hauppauge, NY).

Esterification method. Ester synthesis was carried out in screw-capped test tubes. Unless otherwise specified, 0.12M geraniol, 0.10M acetic acid and 15% (w/w reactants) of SP382 or 2% (w/w reactants) of SP435 lipase were successively added to 2 mL of dry *n*-hexane. Molecular sieves were added to remove water formed during the reaction. All samples were prepared in duplicate and incubated in an orbital shaking water bath at 30°C for 24 h at 200 rpm. A control with no enzyme was incubated under the same conditions.

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 to remove the enzyme and any residual water. Internal standard (*DL*-menthol; 200 μg) was added to each sample. A 1-μL aliquot was analyzed by gas–liquid chromatography with a Hewlett-Packard HP5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a flame-ionization detector. 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. The extent of synthesis was determined from the amount of geraniol consumed in the reaction and quantitated by an on-line computer.

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 and subsequently dried in a desiccator.

RESULTS AND DISCUSSION

The amount of lipase used in a given bioprocess is a crucial economic factor. The concentrations of lipases reported are often too high for any industrial application (21). Enzyme concentrations of 38, 37 and 93% (w/w reactants) have been reported (4,15,17). The effect of varying enzyme concentration on the yield of geranyl acetate by direct esterification is presented in Figure 1. The minimum concentration necessary to achieve maximum yield (96%) was 15% for SP382 and 2% for SP435 lipase. The concentration of SP435 reported here is well below the recommended concentrations for this lipase (5–10%) (19).

The effect of substrate concentrations on the synthesis of flavor esters with *Mucor miehei* (7,14,21,22) and *C. cylindraceae* lipases has been reported (11). Lipases from *M. miehei* were inhibited by increasing concentrations of ethanol (14), butanol (21) and geraniol (7,22). *Candida cylindraceae* lipases were also inhibited by increasing concentrations of ethanol, with an optimum at 0.4M (11). The effects of geraniol and acetic acid concentrations on the

*To whom correspondence should be addressed.

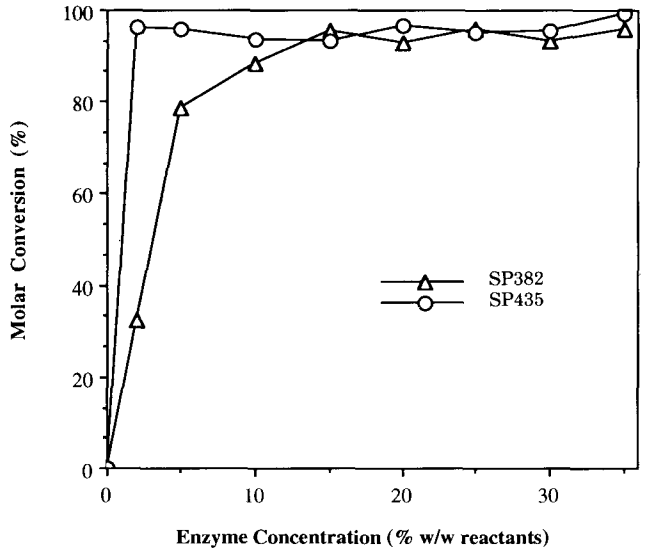
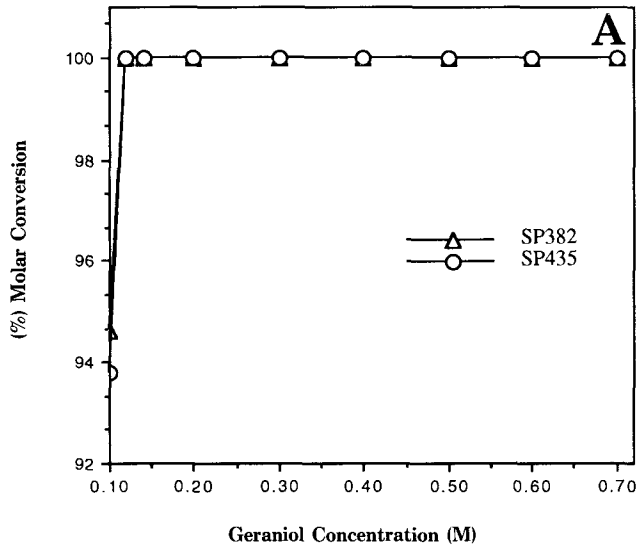


FIG. 1. Effect of enzyme concentration on the direct esterification of geraniol with acetic acid. Samples were prepared by adding 0.1 M geraniol, 0.1M acetic acid to 2 mL dry *n*-hexane and incubated with 0–35% (w/w reactants) of SP382 or SP435.



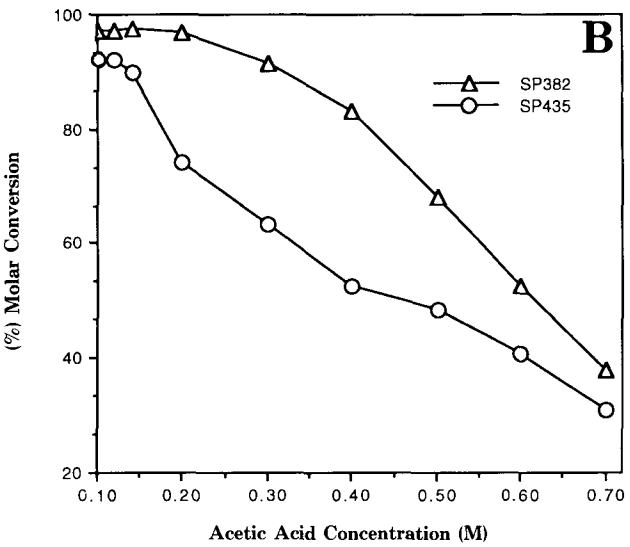


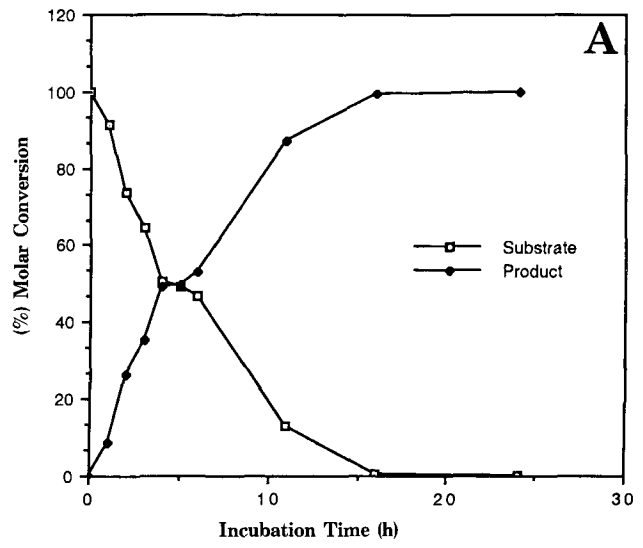
FIG. 2. Effect of substrate concentration on the direct esterification of geraniol with acetic acid. Geraniol concentration effect (A): 0.1M acetic acid was added to 2 mL dry *n*-hexane containing 0.1–0.7M geraniol; 15% (w/w reactants) of SP382 or 2% (w/w reactants) of SP435 was added. Acetic acid effect (B): 0.1M geraniol was used with increasing concentrations of acetic acid.

esterification activities of SP382 and SP435 lipases are shown in Figure 2. Geraniol did not inhibit the esterification activity of SP382 and SP435 (Fig. 2A). In our previous work (20), we found that the esterification activities of *M. miehei* lipases were inhibited by geraniol, whereas those of *C. antarctica* lipases were not. The highest conversion yield was obtained when the geraniol concentration was above 0.1M. Contrary to geraniol, an increase in acetic acid concentration (Fig. 2B) inhibited the activities of the two lipases. The inhibitory effects of acetic acid (4,8,9,17) and butyric acid (11,14,21) on several lipases have been reported. SP382 and SP435 lipases exhibited different degrees of resistance to acetic acid inhibition. SP435 was markedly inhibited at 0.2M and SP382 at 0.5M acetic acid concentrations. Our previous study showed that SP382 was more resistant to acetic acid inhibition than was SP435 (20). However, at 0.7M acetic acid, this difference was not observed; the two lipases retained 34% of their original activity. Based on the results obtained from the effect of enzyme and substrate concentrations, we chose 0.1M acetic acid, 0.12M geraniol, 2% (w/w reactants) of SP435 and 15% (w/w reactants) of SP382 lipase for subsequent experiments.

Figure 3 shows the time course of lipase-catalyzed synthesis of geranyl acetate. SP382 and SP435 lipases gave 99 and 95% molar conversions of geraniol to geranyl acetate, respectively, after 16-h incubation. These yields were higher than the 85% of geranyl acetate reported after 3-d incubation with *M. miehei* (Lipozyme™ IM20) in a transesterification reaction (7). Yields less than 0.5% were obtained after 24-h incubation in an IM20-catalyzed direct synthesis of geranyl acetate (4). Our results represent a significant improvement in the yields of geranyl acetate produced by direct esterification.

The effect of temperature on the synthesis of geranyl acetate by direct esterification is shown in Figure 4. The yield of geranyl acetate over the temperature range studied was greater than 90%. Mutua and Akoh (23) reported an optimal temperature of 55°C for the SP382-catalyzed transesterification of methyl glucoside with methyl oleate in benzene/pyridine mixture. The optimal temperature range in this study was between 35 and 40°C for the two lipases. The reduction in geranyl acetate yield at temperatures higher than 45°C may be due to the volatility of geraniol and/or geranyl acetate rather than the thermolability of the lipases.

The importance of water in enzyme-catalyzed reactions in organic media cannot be overemphasized. Water plays an active role in maintaining the three-dimensional conformation and the active site of enzymes. To favor synthesis over hydrolysis, the control of the water content



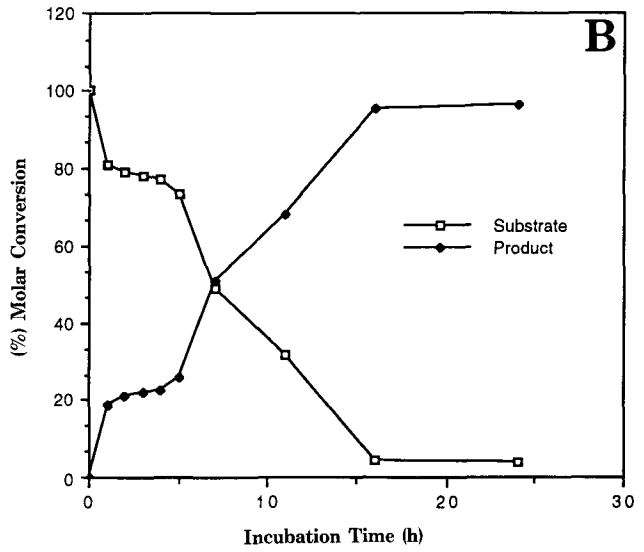


FIG. 3. Time course of lipase-catalyzed direct synthesis of geranyl acetate with SP382 lipase (A) or SP435 lipase (B). Samples were analyzed after 1-, 2-, 3-, 4-, 5-, 6-, 11-, 16- and 24-h incubation.

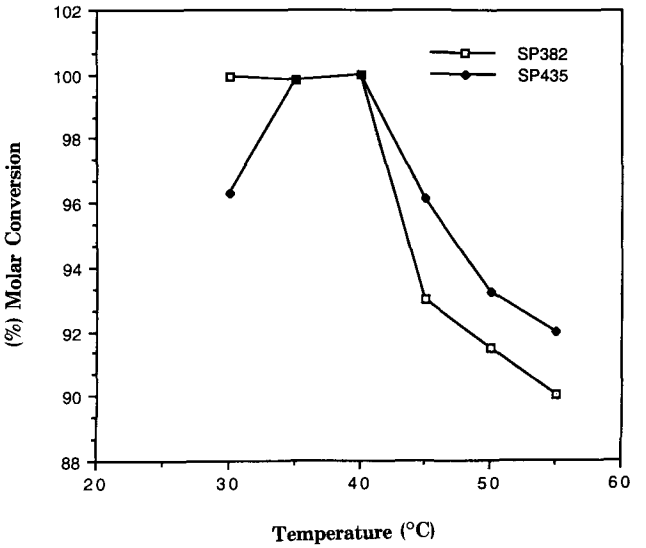


FIG. 4. Effect of temperature on the synthesis of geranyl acetate by direct esterification in *n*-hexane.

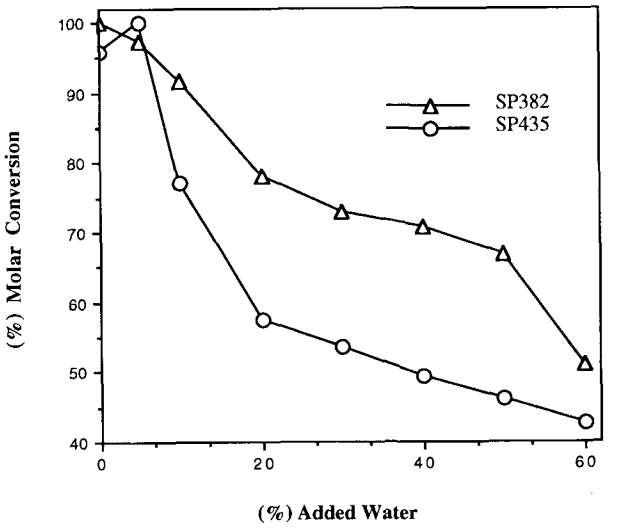


FIG. 5. Effect of added water on the direct esterification of geraniol with acetic acid. Water was added to the hexane before the substrates. Molecular sieves were added after 1-h incubation.

in the reaction medium is vital (24–26). Different levels of water were added to dry *n*-hexane. The water content of solvents was measured with a 684 KF coulometer equipped with a 649 stirrer (Brinkmann Instruments, Inc., Westbury, NY). The original water contents in the lipases and the solvent were 1–2% and 35.5 ppm, respectively. Figure 5 shows the effect of added water on the yield of geranyl acetate. Concentrations of added water, up to 5% (w/w reactants), had little effect on the conversion yields (95–100%). However, as the added water levels increased, the competition between esterification and subsequent hydrolysis of the product became apparent. Addition of 60% (w/w reactants) water caused SP382 and SP435 lipases to lose 50 and 60%, respectively, of their esterification activities.

The polarity of organic solvents used in biocatalysis does affect the enzyme activity (27–29); but the mechanism of action is still controversial (30). The log *P* value of organic solvents is widely used to describe their polarity. *P* value is defined as the partitioning of a given solvent between water and 1-octanol in a two-phase system (24). It is generally recommended to use organic solvents with a log *P* value > 2.0 for biocatalysis (27). Table 1 shows the effect of different organic solvents on the yield of geranyl acetate synthesized by direct esterification. No lipase activity was detected in pyridine (log $P = 0.71$) and tetrahydrofuran (log $P = 0.49$), but in acetonitrile (log $P = -0.33$) there was some activity. We found that solvents with a log *P* value ≥0.85 were suitable for the synthesis of geranyl acetate under our experimental conditions. With the exception of pentane and *iso*-octane, all solvents with a log *P* value >2.0 gave conversion yields >94% when 15% (w/w) SP382 lipase was used. The yields obtained with 2% of SP435 lipase were lower, but the same trend

TABLE 1

Effect of Selected Organic Solvents on the Direct Synthesis of Geranyl Acetate by *Candida antarctica* Lipases (SP382 and SP435)[a]

Solvent	Water content (ppm)	Log *P* value[b]	(%) Molar conversion SP382	SP435
Petroleum ether	124	—	99.32	99.94
iso-Octane	40	4.51	74.56	81.63
n-Hexane	35.5	3.50	95.42	84.25
Cyclohexane	31.3	3.20	94.56	83.54
Pentane	24	3.00	80.70	77.54
Toluene	119	2.50	100.00	94.84
Benzene	156	2.00	96.18	87.30
Diethyl ether	751.7	0.85	69.22	71.19
Pyridine	254.4	0.71	00.00	00.00
Tetrahydrofuran	1400	0.49	00.00	00.00
Acetonitrile	524.7	−0.33	3.37	3.37

[a]Solvents were dried over molecular sieve 4Å.
[b]Source: Reference 27 and *iso*-octane (Ref. 14).

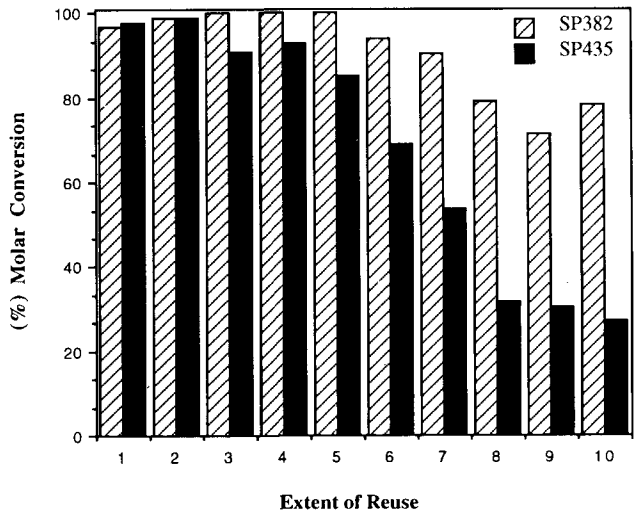


FIG. 6. Effect of enzyme reuse on the direct synthesis of geranyl acetate in *n*-hexane with SP382 and SP435 lipases. After each run, the enzymes were washed with dry hexane, and the solvent was evaporated prior to reuse.

was observed. There was no correlation between the initial water content of the organic solvents used in this study and the conversion yields obtained.

After ten runs (Fig. 6), SP382 and SP435 lipases were still active with yields of 77 and 27%, respectively. We believe that the relatively low conversion yields observed with SP435 after seven runs were due to its low initial concentration (2% w/w), which is below the recommended concentrations (5–10%) (9), and small for the substrate concentrations used in this particular experiment. In addition, SP435 was gradually being lost as we tried to recover the enzyme after each wash. We believe that this problem may not arise in continuous and scale-up processes, which will require large amounts of the SP435 lipase. The ability to reuse enzyme in bioconversions will play a great role in reducing the cost of the overall process.

To the best of our knowledge, this report represents the first comprehensive study of the parameters affecting the synthesis of geranyl acetate by direct esterification with lipases from *C. antarctica*. Most of the reported studies used 1,3-specific lipases from *M. miehei* (Lipozyme™ IM20) (3,7,14,15,17,21,22). This study clearly demonstrated that lipases from *C. antarctica* are suitable for the production of geranyl acetate by direct esterification.

ACKNOWLEDGMENTS

Contributed by the Agricultural Experiment Station, College of Agriculture and Environmental Sciences, The University of Georgia. Research supported by Food Science Research No. 2526 GC 294000 and USDA HATCH Project No. GEO00695.

REFERENCES

1. Clark, B.C. and T.S. Chamblee, in *Off-Flavors in Foods and Beverages*, edited by G. Charalambous, Elsevier Science Publishers B.V., New York, 1992, p. 229.
2. Armstrong, D.W., B. Gillies and H. Yamazaki, in *Flavor Chemistry Trends and Development*, edited by R. Terashini, R.G. Buttery and F. Shahidi, American Chemical Society, Washington, D.C., 1989, p. 105.
3. Rizzi, M., P. Stylos and M. Reuss, *Enzyme Microb. Technol. 14*:709 (1992).
4. Langrand, G., C. Triantaphylides and J. Baratti, *Biotech. Lett. 8*:549 (1988).
5. Triantaphylides, C., G. Langrand, H. Millet, M.S. Rangheard and G. Buono, in *Bioflavour '87*, edited by P. Shreier, Water de Gruyer, New York, 1988, p. 532.
6. Gray, C.J., J.S. Narang and S.A. Barker, *Enzyme Microb. Technol. 12*:800 (1990).
7. Chulalaksananukul, W., J.S. Condoret and D. Combes, *Ibid. 14*:293 (1992).
8. Okumura, S., M. Iwai and Y. Tsujisaka, *Biochim. Biophys Acta 575*:156 (1979).
9. Iwai, M., S. Okumura and Y. Tsujisaka, *Agric. Biol. Chem. 44*:2731 (1980).
10. Marlot, G., G. Langrand, C. Triantaphylides and J. Baratti, *Biotech. Lett. 9*:647 (1985).
11. Gillies, B., H. Yamazaki and D.W. Armstrong, *Ibid. 9*:709 (1987).
12. Takahashi, K., Y. Saito and Y. Inada, *J. Am. Oil Chem. Soc. 65*:911 (1988).
13. Welsh, F., and R.E. Williams, *Enzyme Microb. Technol. 12*:743 (1990).
14. Manjon, A., J.L. Iborra and A. Arocas, *Biotech. Lett. 13*:339 (1991).
15. de Castro, H.F., W.A. Anderson, M. Moo-Young and R.L. Legge, in *Biocatalysis in Non-Conventional Media*, edited by J. Tramper, M.H. Vermúe and H.H. Beeftink, Elsevier Science Publishers B.V., New York, 1992, p. 475.
16. Macrae, A.R., in *Biocatalysis in Organic Syntheses*, edited by J. Tramper, H.C. van der Plas and P. Linko (eds.), Elsevier Science Publishers B.V., New York, 1985, p. 195.
17. Langrand, G., C. Triantaphylides and J. Baratti, *Biotech. Lett. 12*:581 (1990).
18. Nishio, T., T. Chikano and M. Kamimura, *Agric. Biol. Chem. 52*:1208 (1988).
19. Novo Nordisk Bioindustrials Inc., *Product Information*, B 341c-GB December 1992.
20. Claon, P.A., and C.C. Akoh, *Biotech. Lett. 15*:1211 (1993).
21. Borzeix, F., F. Monot and J.P. Vandecasteele, *Enzyme Microb. Technol. 14*:791 (1992).
22. Chulalaksananukul, W., J.S. Condoret and D. Combes, *Ibid. 5*:691 (1993).
23. Mutua, L.N., and C.C. Akoh, *J. Am. Oil Chem. Soc. 70*:43 (1993).
24. Dordick, J.S., *Enzyme Microb. Technol. 11*:194 (1989).
25. Boyce, C.O.L. (ed.), *Novo's Handbook of Practical Biotechnology*, 2nd edn., Novo Industri, Bagsvaerd, 1986, p. 48.
26. Mukherjee, K.D., *Biocatalysis 3*:277 (1990).
27. Laane, C., S. Boeren, K. Vos and C. Veeger, *Biotechnol. Bioeng. 30*:81 (1987).
28. Zaks, A., and A.M. Klibanov, *J. Biol. Chem. 263*:8017 (1988).
29. Gorman, L.A.S., and J.S. Dordick, *Biotechnol. Bioeng. 39*:392 (1992).
30. Narayan, V.S., and A.M. Klibanov, *Ibid. 41*:390 (1993).

[Received November 1, 1993; accepted March 22, 1994]