# Synthesis of *(Z)*-3-Hexen-1-yl Butyrate in Hexane and Solvent-Free Medium Using *Mucor miehei* and *Candida antarctica* Lipases

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**ABSTRACT:** *(Z)*-3-Hexen-1-yl butyrate is an important flavor and fragrance compound as it represents the model of a natural herbaceous (green) note. Two immobilized lipases from *Mucor miehei* (Lipozym IM) and from *Candida antarctica* (Novozym 435) were investigated for their use in the synthesis of *(Z)*-3-hexen-1-yl butyrate by direct esterification in *n*-hexane. To determine optimal conditions for esterification, we examined the following parameters: temperature, amount of lipase, acid/alcohol ratio, and absence of solvent. In *n*-hexane, bioconversion yields reached 95 (after 4 h) and 92% (after 6 h) for, respectively, Lipozym IM [17 (w/w reactants)] and Novozym 435 [2% (w/w reactants)]. In the absence of solvent, at 60°C, Novozym 435-catalyzed esterification afforded the title compound in 80% yield. Up to 250 g (in hexane) and 160 g (without solvent) of ester were easily prepared, in a single operation, at a laboratory scale, in few hours, using 2% (w/w reactants) lipase.
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**KEY WORDS:** Biosynthesis, *Candida antarctica,* direct esterification, *n*-hexane, *(Z)*-3-hexen-1-yl butyrate, lipases, *Mucor miehei*, solvent-free medium.

Low-molecular-weight esters derived from short-chain carboxylic acids (acetates, propionates, butyrates, …) represent an important class of flavoring materials. They are responsible for the fruity odors of many foods and fragrances (1,2). In foodstuff industries, flavor and fragrance loss due to various reasons including manufacturing processes must be compensated for by the addition of aromas. Moreover, there is a growing demand for natural flavors containing green notes represented by C-6 alcohols derivatives. Hence, the biological production of such esters is of much current commercial interest. In this context, the use of immobilized lipases working in nonaqueous media (3–14) has potential since esters produced from natural acids and alcohols may be considered as natural. This mode of production is of great interest, as it would make food industry less dependent on seasonal, climatic, and geographical variations.

The present work deals with the biosynthesis of *(Z)*-3-hexen-1-yl butyrate as a model compound catalyzed by two commercial immobilized lipases from *Mucor miehei* and from *Candida antarctica*. Our purpose was to determine best experimental conditions and procedure to produce rather large amounts of $C_6$ alcohol-derived esters (150 to 250 g), at a laboratory scale, in a single operation as an intermediate step to pilot scale, our ultimate goal being the industrial production from natural acids and alcohols of esters considered to be natural (15–17). The impact of several parameters on the bioreaction was carefully investigated and optimized, i.e., the nature and the amount of enzyme, the substrate concentration, the temperature, and the acid/alcohol ratio. We also investigated *C. antarctica*-catalyzed synthesis of the title compound in the absence of solvent.

## MATERIALS AND METHODS

*Materials.* Lipases from *M. miehei* immobilized on macroporous anion exchange resin Lipozym IM [5–6 Batch Acidolysis Units NOVO (BAUN)·$g^{-1}$, cloned into *Aspergillus oryzae*] and *C. antarctica* immobilized on acrylic resin Novozym 435 [7000 Propyl Laurate Units (PLU)·$g^{-1}$, cloned into *A. oryzae*] were purchased from Novo Nordisk Bioindustrials, Inc. (Bagsvaerd, Denmark). *(Z)*-3-Hexen-1-ol (98% pure) was provided by the Company Naturex (Montfavet, France). Butyric acid was purchased from Aldrich (Steinheim, Germany).

*Esterification method.* Ester synthesis was carried out in a 50-mL two-necked round flask equipped with a condenser topped with a calcium chloride guard tube. For the scale-up, we used either a 500-mL or 2000-mL three-necked round flask equipped with a mechanical stirrer. (i) With Lipozym IM: To dry *n*-hexane (20 mL) were successively added alcohol (0.5 M), butyric acid (0.75 M), and *M. miehei* lipase [17% (w/w reactants)]. The reaction mixture was magnetically stirred (250 rpm) in a thermostated oil bath at 40°C. (ii) With Novozym 435: Alcohol (1.5 M), butyric acid (1.5 M), and *C. antarctica* lipase [2% (w/w reactants)] were added to 20 mL of hexane. The suspension was incubated in a thermostated

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oil bath at 70°C and magnetically stirred at 250 rpm (18). (iii) Esterification in a solvent-free medium was carried out with the same apparatus. The mixture of alcohol (0.12 mole), butyric acid (0.12 mole), and Novozym 435 [2% (w/w reactants)] was incubated at 60°C and magnetically stirred at 250 rpm.

*Chromatography analyses*. Aliquots were periodically withdrawn and analyzed on a Hewlett-Packard gas chromatograph model HP 5890 (Palo Alto, CA) equipped with a capillary column HP INNO WAX (0.4 μm, 50 m × 0.2 mm). Split/splitless injector and mass spectrometer detector were set at 250 and 280°C, respectively. Helium was used as carrier at a flow rate of 15 mL·min$^{-1}$. The temperature gradient was the following: 10°C/min from 50 to 150°C, followed by 15°C/min from 150 to 230°C. *(Z)*-3-Hexen-1-ol and *(Z)*-3-hexen-1-yl butyrate were quantitated relative to (±) linalol as internal standard.

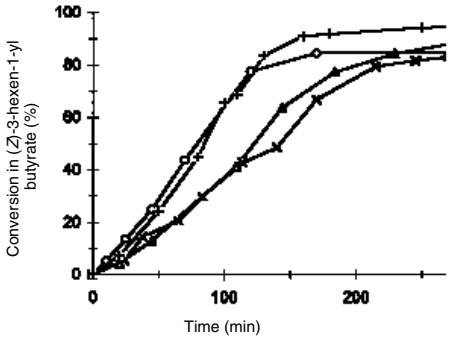


**FIG. 2.** Effect of butyric acid/*(Z)*-3-hexen-1-ol molar ratio on the rate of formation of *(Z)*-3-hexen-1-yl butyrate in hexane (20 mL) in the presence of *Mucor miehei* lipase [17% (w/w reactants)], 40°C; (+) = 0.75 mol·L$^{-1}$ acid/0.5 mol·L$^{-1}$ alcohol ($R$ = 1.5); (○) = 0.5 mol·L$^{-1}$ acid/0.5 mol·L$^{-1}$ alcohol ($R$ = 1); (×) = 0.75 mol·L$^{-1}$ acid/0.75 mol·L$^{-1}$ alcohol ($R$ = 1); (△) = 1.5 mol·L$^{-1}$ acid/1 mol·L$^{-1}$ alcohol ($R$ = 1.5).

## RESULTS AND DISCUSSION

Mucor miehei *lipase-mediated synthesis*. The effect of temperature on the synthesis of *(Z)*-3-hexen-1-yl butyrate by direct esterification is shown in Figure 1A. In accordance with previous reports (4,19,20), the optimal temperature for Lipozym IM was found to be 40°C. In that condition, 92% conversion yield was observed in 3 h compared with 87% in 6.5 h at 25°C.

The amount of lipase is a crucial economic factor for any bioconversion process. The concentrations of lipase reported are often too high to meet industrial requirements (21). Enzyme concentrations of 38 and 93% (w/w reactants) have been reported (7,10). Having as an ultimate goal the development of a large-scale process, we assessed the effect of varying enzyme concentration on the yield of *(Z)*-3-hexen-1-yl butyrate by direct esterification (Fig. 1B). As already noted, the rate of esterification was related to the amount of enzyme involved (9,12,19,21–23). In our hands, the minimum concentration of Lipozym IM necessary to achieve maximum conversion yield (92% in 15 h) was 4% (w/w reactants).

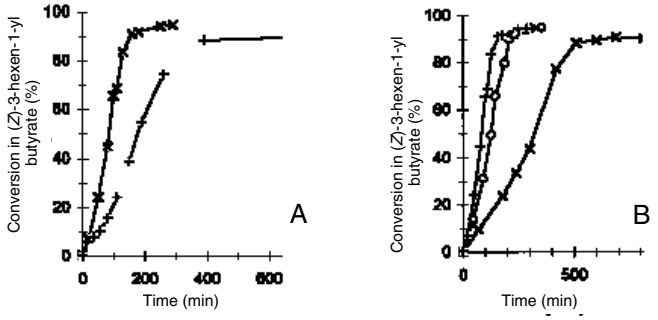


**FIG. 1.** (A) Effect of reaction temperature on the rate of esterification of *(Z)*-3-hexen-1-ol with butyric acid catalyzed by the lipase of *Mucor miehei* [Lipozym IM 17% (w/w reactants)] in hexane (20 mL), 0.75 mol·L$^{-1}$ acid/0.5 mol·L$^{-1}$ alcohol; (x) = 40°C; (+) = 25°C. Lipozym IM: Novo Nordisk, Bagsvaerd, Denmark. (B) Effect of the concentration of Lipozym IM [% (w/w reactants)] on the rate of formation of *(Z)*-3-hexen-1-yl butyrate in hexane (20 mL), 40°C, 0.75 mol·L$^{-1}$ acid/0.5 mol·L$^{-1}$ alcohol; (+) = 17%; (○) = 13%; (×) = 4%.

Two approaches were followed in the study of the effect of substrate concentration. First, we evaluated the effect of increasing the concentration of one of the substrates while keeping the other constant, then the effect of increasing the concentration of both substrates while maintaining the same molar ratio. The effects of substrate molar ratio on both the reaction rate and amount of ester formed are presented in Figure 2, for three different situations: #1, 0.75 M acid/0.5 M alcohol ($R_1$ = 1.5); #2, 0.5 M acid/0.5 M alcohol ($R_2$ = 1); #3, 0.75 M acid/0.75 M alcohol ($R_3$ = 1).

As seen in Figure 2, maximum conversion yield (94 to 95%) and esterification rate (4 h) were obtained with reaction conditions #1 [$R_1$ = 1.5; 0.5 M *(Z)*-3-hexen-1-ol]. Reactions #2 and #3 led to maximum conversion yields of, respectively, 85% (4 h) and 83% (4.5 h). As expected, reaction rate was dependent on substrate concentration. For 0.5 M alcohol, an excess of butyric acid increased both the rate of esterification and maximum conversion yield (#1). The inhibitory effect of butyric acid on Lipozym IM activity was not detected as previously reported (4,6,21). One possible explanation may arise from the nature of the support to which the enzyme is linked. Several reports have emphasized the role that the support may play on the efficiency of the enzyme (4,5,24–27). Several parameters, including the hydrophilic-lipophilic balance (24), the accessibility to the catalytic site, the particle size, pore size, etc. (3,15,24), govern the enzyme activity. In the case of a celite-adsorbed *M. miehei* lipase, Manjón *et al.* (4) noted a significant decrease of lipase activity when butyric acid concentration exceeded 0.4 mol·L$^{-1}$. According to these authors, this behavior should be ascribed to a decrease of the pH of the enzyme aqueous environment. Others have reported (11) that the effectiveness of the lipase from *C. cylindraceae* immobilized on nylon was not affected by butyric acid concentration since the rate of esterification increased steadily up to

2 mol·L$^{-1}$. By keeping constant the butyric acid/*(Z)*-3-hexen-1-ol molar ratio ($R$ = 1), an increase of the concentration of both substrates affected the rate of the reaction. Similarly, a twofold increase of substrate concentrations in the optimal molar ratio situation $R'$ = 1.5 resulted in a reduction of the maximum conversion yield from 95 to 85% (for 4 h reaction). These findings are consistent with published results (4,12,19,21) dealing with *M. miehei* lipase (Lipozym IM). The loss of effectiveness resulting from an increase of substrate concentration may be due either to a substrate inhibition or, more likely, to a modification of the polarity of the medium. Zaks and Klibanov (28) demonstrated that it is the water bound to the enzyme which determines the catalytic activity rather than the total water content. It is generally accepted that the more polar the solvent, the more water will be held in solution instead of bound to the enzyme, with a concomitant decrease of both the stability and activity (28). Best solvents for lipase-mediated esterifications are those which do not withdraw water from the enzyme water monolayer. These are characterized by high log $P$ values (log $P \geq 4$) (29).

Candida antarctica *lipase-mediated biosynthesis*. In view of the excellent results obtained in our laboratory in the acylation of *(Z)*-3-hexen-1-ol by acetic acid using the enzyme of *C. antarctica* (18), we decided to investigate the Novozym 435-mediated esterification of this alcohol. In using the same conditions as those determined for Lipozym IM [17% (w/w reactants)] with Novozym 435, maximum conversion yield (96%) was obtained in 20 min whereas in the same conditions only 7% of ester was produced with Lipozym IM. With the latter, it took 130 min to reach the plateau (92%). These findings substantiate the observations reported by Claon and Akoh (8) and others (20,30).

For safety, economic, technological and environmental reasons, enzyme-mediated direct esterification in a solvent-free medium is of utmost importance especially in the food industry. In the last 6 yr, lipase-mediated direct esterification and transesterification have motivated research on *M. miehei* (9,23,25,31), *C. cylindraceae* (11,25), and *C. antarctica* (13,14,32,33) lipases. For instance, lipase from *C. antarctica* was reported (32) to mediate the transesterification synthesis of geranyl and $\beta$-citronellyl acetate in 94% yield (in 10 h) compared to 74% (in 6 h) for the direct esterification of $\beta$-citronellol by acetic acid (13). However, geranyl butyrate was produced in almost quantitative yield in 7 h at 60°C from a mixture of acid, alcohol (molar ratio 0.87), and 500 mg of *C. antarctica* lipase, i.e., 7% (w/w reactants) (14). In our experiments, the formation of *(Z)*-3-hexen-1-yl butyrate was slower (6 h) and the procedure less efficient (80% yield) in a solvent-free medium than in hexane (92% yield in 2 h).

To determine the influence of the acid/alcohol molar ratio on the course of the direct esterification of *(Z)*-3-hexen-1-ol in hexane, we investigated the following situations: (i) 1.5 mol·L$^{-1}$ acid, 1.5 mol·L$^{-1}$ alcohol, $R$ = 1; (ii) 1.725 mol·L$^{-1}$ acid, 1.5 mol·L$^{-1}$ alcohol, $R$ = 1.15; (iii) 1.875 mol·L$^{-1}$ acid, 1.5 mol·L$^{-1}$ alcohol, $R$ = 1.25; (iv) 2.25 mol·L$^{-1}$ acid, 1.5 mol·L$^{-1}$ alcohol, $R$ = 1.5. In hexane, at 70°C, in the presence of 2% (w/w reactants) of lipase (Fig. 3A), best results (92 to 95% yield in less than 3 h) were obtained for molar ratios $R > 1$ (ii, iii, iv) compared to 88% within the same time when $R$ = 1 (i). In that respect, Novozym 435 behaved similarly to Lipozym IM except that: (i) maximum conversion yield was slightly superior; (ii) neither the diminution of conversion yield nor the increase of reaction rate was observed even when the concentration in butyric acid reached 2.25 mol·L$^{-1}$. Similar observations have been reported for the lipase of *C. cylindraceae* immobilized on nylon (11). (iii) In contrast to what happened when acetic acid was the acylating reagent (18), conversion yield increased from 90 to 95% yield when butyric acid concentration increased from 1.5 to 2.25 mol·L$^{-1}$. (iv) This study allowed us to establish the superiority of the lipase of *C. antarctica* over that of *M. miehei* for the direct esterification of *(Z)*-3-hexen-1-ol. Working with only 2% (w/w reactants) enzyme, conversion yield reached 85% within 1 h compared to 30% using 17% (w/w reactants) of Lipozym IM.

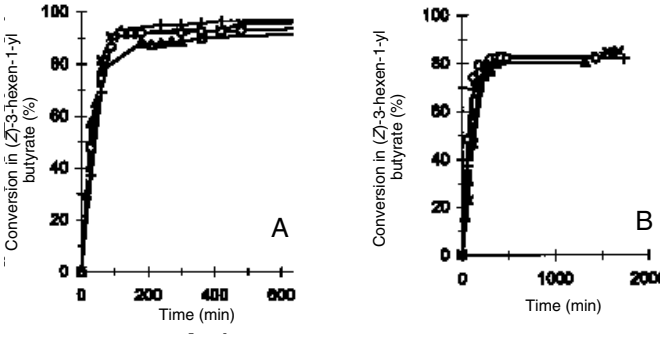


**FIG. 3.** (A) Effect of butyric acid/*(Z)*-3-hexen-1-ol molar ratio on the rate of formation of *(Z)*-3-hexen-1-yl butyrate in hexane (20 mL) in the presence of *Candida antarctica* lipase [2% (w/w reactants)], 70°C; (△) = 1.5 mol·L$^{-1}$ acid/1.5 mol·L$^{-1}$ alcohol ($R$ = 1); (○) = 1.725 mol·L$^{-1}$ acid/1.5 mol·L$^{-1}$ alcohol ($R$ = 1.15); (+) = 1.875 mol·L$^{-1}$ acid/1.5 mol·L$^{-1}$ alcohol ($R$ = 1.25); (×) = 2.25 mol·L$^{-1}$ acid/1.5 mol·L$^{-1}$ alcohol ($R$ = 1.5). (B) Effect of butyric acid/*(Z)*-3-hexen-1-ol molar ratio on the rate of formation of *(Z)*-3-hexen-1-yl butyrate in a solvent-free medium in the presence of *C. antarctica* lipase [2% (w/w reactants)], 60°C; (△) = 0.12 mol acid/0.12 mol alcohol ($R$ = 1); (○) = 0.138 mol acid/0.12 mol alcohol ($R$ = 1.15); (+) = 0.15 mol acid/0.12 mol alcohol ($R$ = 1.25); (×) = 0.18 mol acid/0.12 mol alcohol ($R$ = 1.5).

Without solvent, whatever the acid/alcohol molar ratio, maximum conversion yield was 82% (Fig. 3B). The latter was reached after 8 h ($R$ = 1), 5 h ($R$ = 1.15; $R$ = 1.25), and 6 h ($R$ = 1.5)

Very few reports deal with the extrapolation of a biosynthetic process from the laboratory (≈20 mL) to semipreparative (100 to 200 mL) (10,34) and/or to large scale (1 to 5 L) (22). We investigated the effect of the reaction volume by multiplying by 12.5 (250 mL) and 50 (1000 mL) all parameters, standard experiments being those performed in a 20-mL volume. To achieve such experiments, we used a "reflux" rotary evaporator equipped with a graduated tube instead of the conventional receiving flask. The advantages of using such apparatus are: (i) It ensures a gentle although efficient dispersion of the immobilized lipase. Thus we noted an increase of the lipase lifetime. The enzyme could be reused several times

without a significant loss of efficiency (Bourg-Garros, S., N. Razafindramboa, and A.A. Pavia, unpublished results). This is probably due to a lower degree of breakdown of the solid material compared with that observed when the medium is magnetically stirred. (ii) It represents an intermediate step between the laboratory and the pilot-scale which was our next goal, especially since the apparatus to be used at the pilot plant was a rotary-type device.

In contrast to results reported by Langrand *et al.* (10) dealing with *Rhizopus arrhizus*-mediated esterification of geraniol, a multifold increase of reaction volume was not accompanied by a diminution of the conversion yield (Fig. 4A). The latter was 91, 90, and 94%, respectively, for 20-, 250-, and 1000-mL experiments. When the same approach was applied to a solvent-free medium system, using the same apparatus, we obtained similar results except that the maximum conversion yield did not exceed 77% (5 to 6 h) (Fig. 4B). From these results, we may anticipate that the extrapolation of such experimental conditions and procedures to large-scale preparation should be straightforward.

The ability of *C. antarctica* lipase [10% (w/w reactants)] to synthesize other flavor esters was demonstrated at the 500-mL scale in hexane using equimolar concentrations (1.5 $mol \cdot L^{-1}$) of acetic, butyric and isovaleric acids and *(Z)*-3-hexen-1-ol. The reaction was performed at 70°C. After 30 min, conversion yields were 31% (acetic acid), 88% (butyric acid), and 86% (isovaleric acid). Maximum conversion yields were, respectively, 92% (3 h), 86% (1 h), and 93% (2 h). However, the reaction is slower with acetic acid than with longer-chain acids as already observed by several authors (4,7,8,35). Compared to the lipase from *M. miehei* which is known to display better affinity and higher effectiveness for long-chain carboxylic acid (4,7,8), the *C. antarctica* lipase is well suited to perform biosynthetic esterification of alcohols by short-chain acids. In all cases, approximately 92 to 95% conversion yields were obtained in accordance with results published by Claon and Akoh (8). It is noteworthy that substrate concentrations employed in this work are 6 to 15 times higher than those usually reported in the literature (4,7,8).

From the comparative study of the ability of two commercial immobilized lipases from *M. miehei* (Lipozym IM) and *C. antarctica* (Novozym 435) to catalyze direct esterification of *(Z)*-3-hexen-1-ol by butyric acid, we established the superiority of Novozym 435 in terms of both reaction rate and conversion yield. Best results were obtained in hexane whereas solvent-free medium biosyntheses proved less efficient and were slower. It is noteworthy that experimental conditions set up in this work are significantly different from those reported to date in the literature. In hexane, substrate concentrations were approximately 15 times higher than those reported whereas the amount of enzyme was five times smaller (8). Without solvent, our experiments were performed with amount of substrate 4 to 40 times higher and amount of lipase 3.5 times smaller (14). We were able to extrapolate experimental condition and parameters to large-scale laboratory syntheses: 250 g of *(Z)*-3-hexen-1-yl butyrate was easily prepared in one run, in a few hours with a yield of >90%.

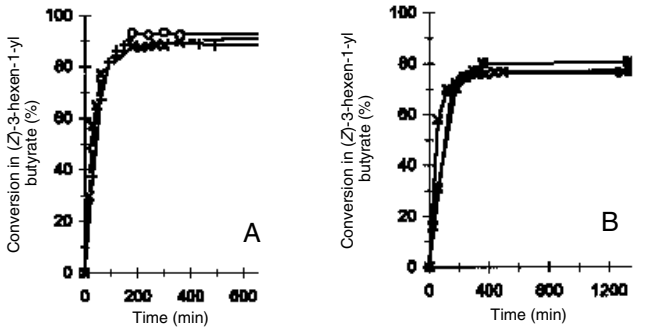


**FIG. 4.** (A) Effect of increasing the reaction volume on the rate of formation of *(Z)*-3-hexen-1-yl butyrate in hexane in the presence of *Candida antarctica* lipase [2% (w/w reactants)], 1.5 $mol \cdot L^{-1}$ acid/1.5 $mol \cdot L^{-1}$ alcohol, 70°C; (×) = 20 mL; (+) = 250 mL; (○) = 1000 mL. (B) Effect of increasing the reaction volume on the rate of formation of *(Z)*-3-hexen-1-yl butyrate, in a solvent-free medium, in the presence of *C. antarctica* lipase [2% (w/w reactants)], 70°C; (×) = 0.12 mol; (+) = 0.24 mol; (○) = 1.20 mol.

## REFERENCES


1. Bauer, K., D. Garbe, and H. Surburg, *Common Fragrance and Flavor Materials,* VCH Publishers, New York, 1990.
2. Richard, H., and J.L. Multon, *Les arômes alimentaires,* Tec & Doc-Lavoisier, Paris, 1992.
3. Vázquez Lima, F., D.L. Pyle, and J.A. Asenjo, Factors Affecting the Esterification of Lauric Acid Using an Immobilized Biocatalyst: Enzyme Characterization and Studies in a Well-Mixed Reactor, *Biotechnol. Bioeng. 46*: 69–79 (1995).
4. Manjón, A., J.L. Iborra, and A. Arocas, Short-Chain Flavour Ester Synthesis by Immobilized Lipase in Organic Media, *Biotechnol. Lett. 13*:339–344 (1991).
5. Marlot, C., G. Langrand, C. Triantaphylides, and J. Baratti, Ester Synthesis in Organic Solvent Catalyzed by Lipases Immobilized on Hydrophilic Supports, *Ibid. 7*:647–650 (1985).
6. Gillies, B., H. Yamazaki, and D.W. Armstrong, Production of Flavor Esters by Immobilized Lipase, *Ibid. 9*:709–714 (1987).
7. Langrand, G., N. Rondot, C. Triantaphylides, and J. Baratti, Short-Chain Flavor Esters Synthesis by Microbial Lipases, *Ibid. 12*:581–586 (1990).
8. Claon, P.A., and C.C. Akoh, Enzymatic Synthesis of Geraniol and Citronellol Esters by Direct Esterification in *n*-Hexane, *Ibid. 15*:1211–1216 (1993).
9. Krmelj, V., M. Habulin, and Z. Knez, Synthesis of Oleyl Oleate with Immobilized Lipase from *Mucor miehei*, *Fat Sci. Technol. 97*:324–326 (1995).
10. Langrand, G., C. Triantaphylides, and J. Baratti, Lipase-Catalyzed Formation of Flavour Esters**,** *Biotechnol. Lett. 10*: 549–554 (1988).
11. Carta, G., J.L. Gainer, and M.E. Gibson, Synthesis of Esters Using a Nylon-Immobilized Lipase in Batch and Continuous Reactors, *Enzyme Microb. Technol. 14*:904–910 (1992).
12. Razafindralambo, H., C. Blecker, G. Lognay, M. Marlier, J.P. Wathelet, and M. Severin, Improvement of Enzymatic Synthesis Yields of Flavour Acetates: The Example of Isoamyl Acetate, *Biotechnol. Lett. 16*:247–250 (1994).
13. Fonteyn, F., C. Blecker, G. Lognay, M. Marlier, and M. Severin, Optimization of Lipase-Catalyzed Synthesis of Citronellyl Acetate in Solvent-Free Medium, *Ibid. 16*:693–696 (1994).

14. Oguntimein, G.B., W.A. Anderson, and M. Moo-Young, Synthesis of Geraniol Esters in a Solvent-Free System Catalyzed by *Candida antarctica* Lipase, *Biotechnol. Lett*. *17*:77–82 (1995).
15. Dordick, J.S., Enzymatic Catalysis in Monophasic Organic Solvents, *Enzyme Microb. Technol. 11*:194–211 (1989).
16. Gatfield, I.L., Production of Flavor and Aroma Compounds by Biotechnology, *Food Technol*. *42*:110–121 (1988).
17. Wasserman, B.P., T.J. Monville and E.L. Korweck, Food Biotechnology, *Ibid*. *42*:133–146 (1988).
18. Bourg-Garros, S., N. Razafindramboa, and A.A. Pavia, Optimization of Lipase-Catalyzed Synthesis of *(Z)*-3-Hexen-1-yl Acetate by Direct Esterification in Hexane and in a Solvent-free Medium, *Enzyme Microb. Technol.,* 1997*,* in press.
19. Monot, F., F. Borzeix, M. Bardin, and J.P. Vandecasteele, Enzymatic Esterification in Organic Media: Role of Water and Organic Solvent in Kinetics and Yield of Butyl Butyrate Synthesis, *Appl. Microbiol. Biotechnol. 35:*759–765 (1991).
20. Novo Nordisk Bioindustrial Inc., *Product Information B 347c-GB 200,* Bagsvaerd, Denmark, December 1992.
21. Borzeix, F., F. Monot, and J.P. Vandecasteele, Strategies for Enzymatic Esterification in Organic Solvents: Comparison of Microaqueous, Biphasic and Micellar System, *Enzyme Microb. Technol. 14*:791–797 (1992).
22. Bloomer, S., P. Adlercreutz, and B. Mattiasson, Kilogram-Scale Ester Synthesis of Acyl Donor and Use in Lipase-Catalyzed Interesterifications, *J. Am. Oil Chem. Soc. 69*:966–973 (1992).
23. Pecnik, S., and K. Knez, Enzymatic Fatty Ester Synthesis, *Ibid. 69*:261–265 (1992).
24. Kawamoto, T., K. Sonomoto, and A. Tanaka, Esterification in Organic Solvents: Selection of Hydrolases and Effects of Reaction Conditions, *Biocatalysis 1*:137–145 (1987).
25. Gray, C.J., J.S. Narang, and S.A. Barker, Immobilization of Lipase from *Candida cylindraceae* and Its Use in the Synthesis of Menthol Esters by Transesterification, *Enzyme Microb. Technol. 12*:800–807 (1990).
26. Muderhwa, J., M. Pina, and J. Graille, Aptitude à la Transestérification de Quelques Lipases Régiosélectives 1-3 (I), *Oléagineux 43*:385–391 (1988).
27. Basri, M., K. Ampon, W.M.Z. Wan Yunus, C.N.A. Razak, and H.B. Salleh, Enzymatic Synthesis of Fatty Esters by Hydrophobic Lipase Derivatives Immobilized on Organic Polymer Beads, *J. Am. Oil Chem. Soc. 72*:407–411 (1995).
28. Zaks, A., and A.M. Klibanov, The Effect of Water on Enzyme Action in Organic Media, *J. Biol. Chem. 263*:8017–8021 (1988).
29. Laane, C., S. Boeren, K. Vos, and C. Veeger, Rules for Optimization of Biocatalysis in Organic Solvents, *Biotechnol. Bioeng. 30*:81–87 (1987).
30. Novo Nordisk Bioindustrial Inc., *Product Information B 665a-GB 200,* Bagsvaerd, Denmark, December 1992.
31. Habulin, M., V. Krmelj, and Z. Knez, Synthesis of Oleic Acid Esters Catalyzed by Immobilized Lipase, *J. Agric. Food Chem. 44*:338–342 (1996).
32. Claon, P.A., and C.C. Akoh, Lipase-Catalyzed Synthesis of Primary Terpenyl Acetates by Transesterification: Study of Reaction Parameters, *Ibid. 42*:2349–2352 (1994).
33. Coulon, D., M. Giradin, B. Rovel, and M. Ghoul, Comparison of Direct Esterification and Transesterification of Fructose by *Candida antarctica* Lipase, *Biotechnol. Lett. 17*:183–186 (1995).
34. Bloomer, S., P. Adlercreutz, and B. Mattiasson, Facile Synthesis of Fatty Acid Esters in High Yields, *Enzyme Microb. Technol. 14*:546–552 (1992).
35. Nishio, T., T. Chikano, and M. Kamimura, Ester Synthesis by the Lipase from *Pseudomonas fragi* 22.39B, *Agric. Biol. Chem. 52*:1203–1208 (1988).

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