Parameters Affecting the Synthesis of Geranyl Butyrate by Esterase 30,000 from *Mucor miehei*

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ABSTRACT: The factors affecting the synthesis of geranyl butyrate by esterase 30,000 of *Mucor miehei* were studied in a solvent-free system. The effects of substrate molar ratio, temperature, agitation speed, and initial addition of water were investigated. The equimolar ratio was most interesting for ester production in batch. There were no diffusion limitations, and the reaction could be realized at low agitation. The catalytic activity of the enzyme was irreversibly deactivated at 60°C, and the initial addition of water decreased the rate of conversion after 75 h of reaction. The enzyme activity increased with increased linear chainlength of the acid and was also affected by the alcohol structure. Esterase 30,000 gave the highest conversion of butyric acid with hexanol and terpenic alcohols (citronellol, nerol) and the lowest with the secondary alcohol (2-hexanol). Finally, five other industrial enzymatic preparations were investigated for their ability to synthesize geranyl butyrate and to hydrolyze olive oil. We observed, for the lipase from *Rhizopus javanicua,* that there is no relationship between hydrolytic and synthetic activities; this example shows that the hydrolytic lipase activity data cannot predict the capability of lipases in esterification reactions.
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KEY WORDS: Esterase, esterification, geranyl butyrate, hydrolytic activity, lipases.

Enzymes are specific biocatalysts that catalyze chemical reactions under mild conditions and reduce wastes (1). For these reasons, enzymes are used in industrial applications in several domains. Lipases or triacylglycerol acylhydrolases (E.C. 3.1.1.3) are widely used biocatalysts in the industry. They catalyze the reversible reaction of acylglyceride hydrolysis in the water/oil interface, and they are found in animals, plants, and microorganisms (2,3). Under certain conditions, lipases/esterases can catalyze ester synthesis. This reaction takes place in a microaqueous system in which water activity is low but sufficient to ensure good enzyme functioning. The reaction can be summarized by the following equation:

$$R_1\text{-COOH} + R_2\text{-OH} \underset{\text{Esterification} \rightarrow}{\overset{\leftarrow \text{ Hydrolysis}}{\leftrightarrow}} R_1\text{-COOR}_2 + H_2O$$

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Lipases are used to produce emulsifiers by glycerolysis of marine oils (4) and to synthesize polyglycerol-fatty acid esters (5). The latter are nonionic biodegradable surfactants used in various industrial applications. Solvents and detergents can also be produced by transesterification of rapeseed oil and 2-ethyl-1-hexanol (6). Like other investigators (7–10), we were also interested in the use of lipases to catalyze the biosynthesis of natural flavor components, such us geraniol and citronellol esters, by direct esterification between terpenic alcohols and short-chain acids (C_2–C_6). These esters are essential oil components used in the food, cosmetic, and pharmaceutical industries. The seal "natural" is an important commercial argument because of the development of an interest in "green" products. Lipases used in these applications are of microbial and fungal origins. The main strains are *Mucor miehei, Pseudomonas fluoresens, Rhizopus arrhesuis, R. niveus,* and *Candida cylindracea*. These lipases are used in impure powder form, soluble or immobilized onto a support. The immobilized form is used more because it is more stable and easier to recover (11–13).

Two types of liquid reaction media are usually used for the enzymatic synthesis of esters: the microaqueous medium contains an organic solvent in which the reactants are dissolved with a small quantity of water (14,15), and the micellar medium differs from the previous medium by the addition of a surfactant (16,17). Lipases can function also in the presence of high reactant concentrations in a system without organic solvents. In this system, the reaction medium is biphasic, composed of the co-soluble substrates in the liquid phase and the enzymatic preparation in the solid phase. This system presents the following advantages: (i) the small reaction volume, which allows a higher quantity of product; (ii) suppression of the problem of toxicity and flammability of organic solvents; and (iii) product purification conditions are simplified (18,19).

In this work, we have studied the parameters that affect the production of geranyl butyrate by esterase 30,000 of *M. miehei* in a solvent-free system. We have studied, in particular, the effects of substrate molar ratio (alcohol/acid) while keeping the total mass of the reaction mixture constant, stability of the enzymatic preparation at high temperature, diffusion limitations by testing the effect of agitation speed, and the effect of initial addition of water. The effects of acid

chainlength and alcohol structure on the enzyme activity were also studied. Finally, we have compared the capability of other industrial enzymes to synthesize geranyl butyrate in a solvent-free system and to hydrolyze olive oil.

MATERIALS AND METHODS

Enzymes. Lipase/esterase from *M. miehei*, esterase 30,000 in powder form was provided by Gist Brocades (Seclin, France). Lipases from different microorganisms were supplied by Biocatalysts LTD (Pontypridd, United Kingdom); they came from *C. cylindracea, P. fluoresens, R. javanicua, R. niveus,* and *115 P* from porcine pancreas. These enzymes were in powder form, and they were used without further purification.

Chemicals. Butyric acid, valeric acid, propionic acid, geraniol (*trans* 3, *cis* 7-dimethyl-2,6-octadien-1-ol), nerol (*cis* 3,7-dimethyl-2,6-octadien-1-ol), and hexanol were provided by Fluka Chemie (France), 2-hexanol was obtained from Merck (Darmstadt, Germany), citronellol (*cis* 7-dimethyl-2,6-octan-1-ol), *cis*-3-hexenol, caproic acid, and olive oil (50%) emulsion were obtained from Sigma (France), acetic acid was provided by Carlo Erba Reagenti (Italy), geranyl butyrate was supplied by PCAS (La Vigne aux Loups, France). All chemicals were of analytical grade.

Esterification. Esterification reactions were carried out in screw-capped flasks that contained 1 g of alcohol and acid in various substrate molar ratios and various quantities of crude enzymatic preparation, with and without addition of water. The flasks were incubated in an orbital shaker at 37°C and 250 rpm. Control experiments were conducted without enzyme.

At precise intervals of time, samples were taken and diluted in hexane that contained 5% hexanol as an internal standard, and they were analyzed by gas chromatography. The conversion yield was calculated on the basis of the limited substrate.

Analysis. The reaction was followed by measuring the quantity of acid remaining and the ester formed by capillary gas chromatography (Carlo Erba, Milano, Italy), equipped with a flame-ionization detector (FID). The column was 25 QC2/.BP21-0.25 (SGE, France), the oven temperature was maintained at 50°C and then increased to 200°C at a rate of 15°C/min. The injector temperature was fixed at 280°C, and the detector temperature at 240°C.

Lipase hydrolytic activity determination. Hydrolytic activity was determined according to Sigma Chemical Co. Bulletin No. 800 with 50% olive oil emulsion as substrate, which is based on the method of Tietz and Fiereck (20). The reaction was carried out at 37°C at pH 7 for 3 h. The free fatty acids liberated were titrated with 0.05 M NaOH and phenolphthalein as indicator. One unit of lipase activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of free acid per min under the above conditions.

Protein content. Protein content was determined by the method of Lowry *et al.* (21).

RESULTS AND DISCUSSION

A preliminary study of the effect of enzyme quantity showed that the amount of ester produced increased when the enzyme quantity was increased. This shows that the reaction is kinetically controlled by the enzyme, and there are no diffusion limitations (22).

Effect of alcohol/acid molar ratio R. When the alcohol/acid molar ratio was decreased while keeping the total mass of the reaction mixture constant, the conversion yield at the end of the esterification reaction decreased (Fig. 1A). Thus, when the acid is the major component of the reaction medium, the conversion yield is systematically lower than the one observed in the reverse situation; this was also reported by a previous study (22). However, if the quantity of produced ester is considered, the alcohol/acid equimolar ratio ($R = 1$), while keeping the total mass of the reaction mixture constant, proves to be the most favorable for ester production in batch (Fig. 1B), although the conversion yield is not maximal.

To study the effects of certain parameters during the first 75 h of reaction, the following operating conditions were chosen as standard conditions: $R = 1.4$, temperature = 37°C, and agitation speed = 250 rpm.

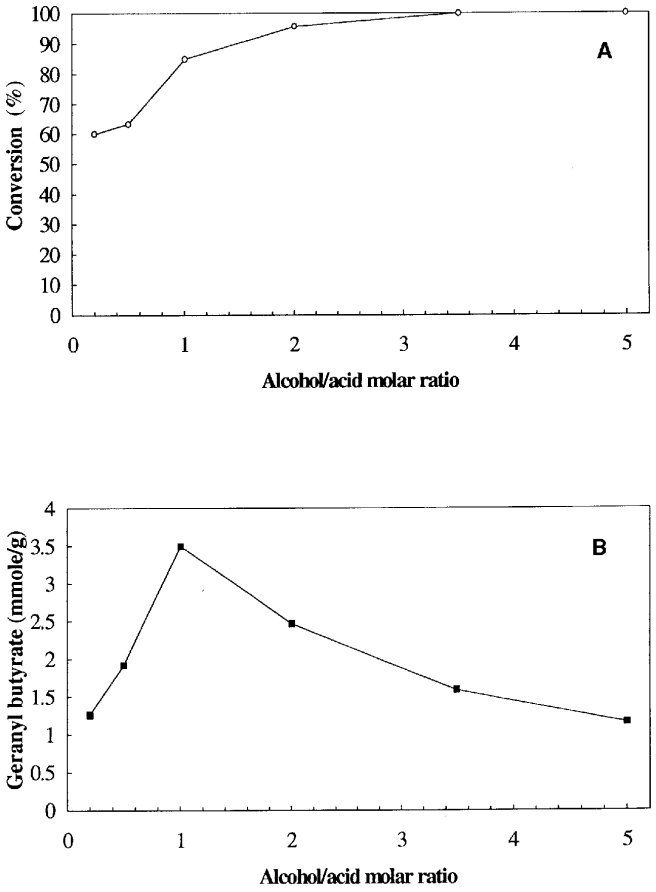

FIG. 1. Effect of substrate molar ratio on the synthesis of geranyl butyrate with 100 mg of esterase 30,000, at 37°C and 250 rpm. (A) The conversion yield (%) at 144 h, (B) ester produced in mmol/g at 144 h.

Effect of temperature. The influence of temperature on the synthesis of geranyl butyrate was investigated at different temperatures: 37, 45, and 60°C. Figure 2 shows the evolution of ester quantity at different temperatures as a function of time. The kinetic profile modifications for the reactions carried out at 45 and 60°C, compared to the kinetic profile observed at 37°C, were the result of the thermal activation of the reaction and the progressive denaturation of proteins by heating. The thermal activation of the reaction is visible during the first 10 h. Thereafter, thermal deactivation takes place progressively, and the quantity of ester produced at the end of the reaction decreases when the temperature goes from 37 to 60°C. The effect of temperature depends on the experimental conditions, such as time of heating, presence of effectors, reactants, products, and water. Razafindralambo *et al.* (23) reported for the same enzyme of *M. miehei* that the optimal temperature to synthesize isoamyl acetate in heptane was 45°C.

To determine if denaturation of the enzyme by heating is reversible, the two following experiments were carried out: Firstly, the reaction was carried out at 60°C for the first 10 h; then the temperature was decreased to 37°C, and the reaction was followed up to 75 h. Secondly, the reaction was carried out at 60°C for the first 35 h; then the temperature was decreased to 37°C, and the reaction was followed up to 75 h. Figure 3 illustrates the kinetic profiles of the reactions for different temperature conditions. The kinetics of the reaction at 60°C for the first 35 h and at 37°C during the rest of the time were practically similar to that carried out totally at 60°C. This can be explained by an irreversible thermal denaturation of the enzyme, because the catalytic activity was not restored when the temperature was decreased to 37°C. When the reaction was carried out at 60°C for only the first 10 h and at 37°C during the rest of time, one sees thermal activation at the beginning of the reaction and a persistence of the residual enzymatic activity when the temperature decreases to 37°C. This shows the progressive character of the thermal enzyme deactivation.

Moreover, to study the direct or indirect progressive and irreversible character of the thermal deactivation of the used biocatalyst, the following experiments were done. Three lots of the crude enzymatic preparation were incubated at 60°C for 24 h. The first lot was incubated alone, the second was incubated in the presence of geraniol, and the third was incubated in the presence of butyric acid. Then, the reaction mediums were completed by the addition of both substrates to the first lot, butyric acid to the second lot, and geraniol to the third lot, and the three reactions were followed at 37°C up to 75 h (see Table 1). Incubation of the enzyme alone at 60°C did not provoke a decrease of the enzymatic activity. However, the enzymatic preparation incubated in the presence of butyric acid was totally denatured; indeed, the conversion yield obtained in 75 h was on the order of that obtained without enzyme (about 3%). For the enzymatic preparation incubated in the presence of geraniol, an increase of the initial rate was found, which is probably due to fixation of geraniol on the enzymatic site. However, this acceleration was followed by a decreased of enzymatic activity. Inactivation of the enzyme is due to the presence of reactants coupled to the temperature. Indeed, incubation of the enzyme with butyric acid at 37°C (not presented in Table 1) did not inhibit the enzyme totally, and the conversion yield obtained in 75 h was about 8%.

Similar results were observed by Monot *et al.* (24). They tested the stability of the same enzyme in hexane with and without 1 g/L of water at 69°C and found that the initial activity of the enzyme heated in hydrated hexane was lower than that of the enzyme heated in dehydrated hexane. How-

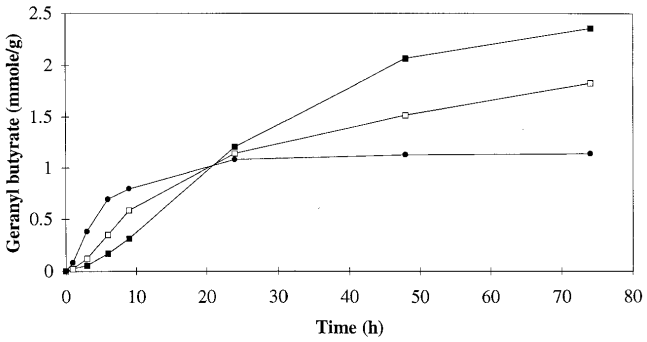

FIG. 2. Effect of temperature on the synthesis of geranyl butyrate with 80 mg of esterase 30,000. Alcohol/acid molar ratio $R = 1.4$, and 250 rpm (■, 37°C; □, 45°C; and ●, 60°C).

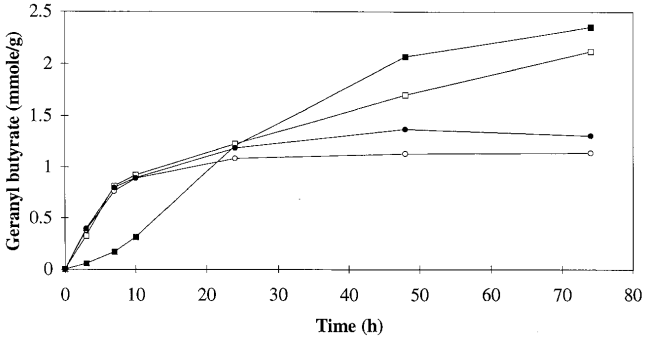

FIG. 3. Effect of heating time of the enzyme with 80 mg of esterase 30,000. Alcohol/acid molar ratio $R = 1.4$, and 250 rpm (■, 37°C; ○, 60°C; □, 60°C when 0 h < time < 10 h and 37°C when 10 h < time < 75 h; and ●, 60°C when 0 h < time < 35 h and 37°C when 35 h < time < 75 h).

TABLE 1
Effect of Enzyme Heating at 60°C in the Presence of Substrates[a]

Incubation of reaction medium at 60°C	Initial rate (mmol/g/h)	Conversion yield (%)
Enzyme + butyric acid	0.003	3
Enzyme + geraniol	0.067	46.7
Enzyme	0.024	81

[a]Alcohol/acid molar ratio $R = 1.4$, 80 mg of esterase 30,000, at 37°C and 250 rpm.

ever, the activity was completely restored after about 3 h of reaction. This restoration phenomenon was not observed in our case when the enzyme was incubated in the presence of butyric acid, which induced an irreversible modification of the enzyme conformation.

Effect of agitation. The reaction system studied was composed of two phases: liquid, containing two reactants, and solid, containing the crude enzymatic powder in which the enzyme was surrounded by unknown additives (sugars, salts). In this system, the kinetic reaction can be affected by diffusion limitations of substrates toward the enzyme. The possible influence of diffusion limitations on the kinetics of synthesis of geranyl butyrate was investigated by testing different agitation speeds (0, 90, 150, and 250 rpm). Figure 4 shows the evolution of ester vs. time for different agitation speeds. It is clear that variation of agitation has no important effect on the esterification reaction because the initial rates obtained at 0, 45, 90, and 150 rpm were constant around 0.042 mmol/g/h. However, the initial rate obtained at 250 rpm was 0.020 mmol/g/h. This decrease in the initial rate may be due to the dispersion of the enzyme in the reaction medium; the enzyme cannot be aggregated, so its hydration becomes difficult (25). In this situation, the reaction rate was not limited by the rate of external diffusion, so the kinetic velocity was lower than the substrate's external diffusion.

Effect of initial addition of water. In the esterification reaction, water constitutes an important factor because it has an effect on the thermodynamic equilibrium of the chemical reaction. Because it is reversible, water favors hydrolysis and inhibits esterification. On the other hand, water activates the enzyme and permits good functioning of the catalyst (26). During our study, we have carried out the esterification reaction with geraniol and butyric acid without initial addition of water. The yield conversion obtained at the end of the reaction was significant, so the water existing in the substrates (0.4% w/w) and enzyme preparation (5% w/w), as well as the water produced during the reaction, was sufficient to moisten the enzyme and to render it active. In this work, we have studied the influence of the initial addition of increasing quantities of water on the esterification reaction. We have added in the reaction medium 0.1, 0.2, 0.5, and 1% of 10^{-2} M phosphate buffer pH 7.5 (w/w). The addition of water increases the initial rate and decreases the quantity of ester produced at the end of the reaction (Fig. 5). Indeed, the initial rate obtained without the addition of buffer was 0.020 mmol/g/h, and the quantity of ester produced at the end of the reaction was 2.35 mmol/g; however, when 1% of buffer was added, the initial rate obtained was 0.046 mmol/g/h, and the quantity of ester produced at the end of the reaction was 1.3 mmol/g. These results confirm that the enzyme preparation and substrates contain sufficient water to ensure enzyme hydration.

Effect of acid carbon chainlength. The influence of acid chainlength on the activity of esterase 30,000 was studied

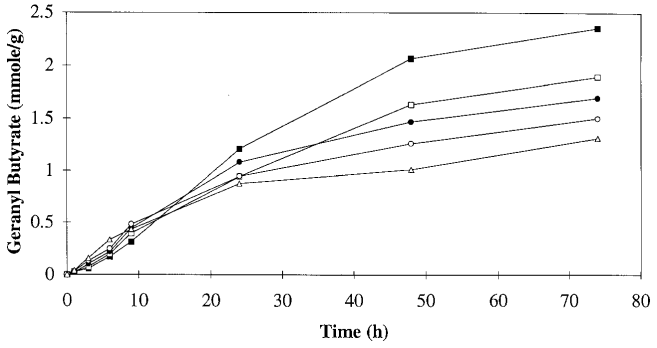

FIG. 5. Effect of addition of water on the synthesis of geranyl butyrate with 80 mg of esterase 30,000. Alcohol/acid molar ratio $R = 1.4$, at 37°C and 250 rpm (■, 0% water; □, 0.1% water; ●, 0.2% water; ○, 0.5% water, and ▲, 1% water).

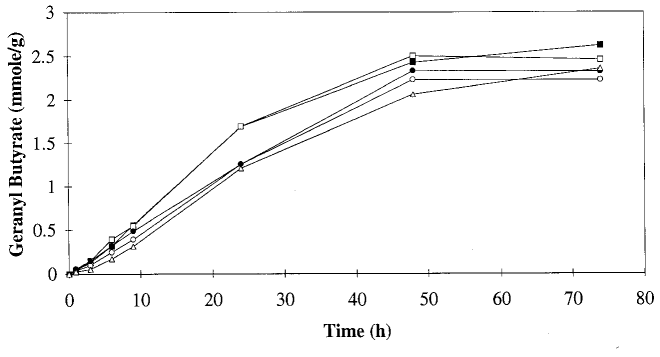

FIG. 4. Effect of agitation on the synthesis of geranyl butyrate with 80 mg of esterase 30,000. Alcohol/acid molar ratio $R = 1.4$, at 37°C (■, 0 rpm; ❑, 45 rpm; ○, 90 rpm; ●, 150 rpm; and △, 250 rpm).

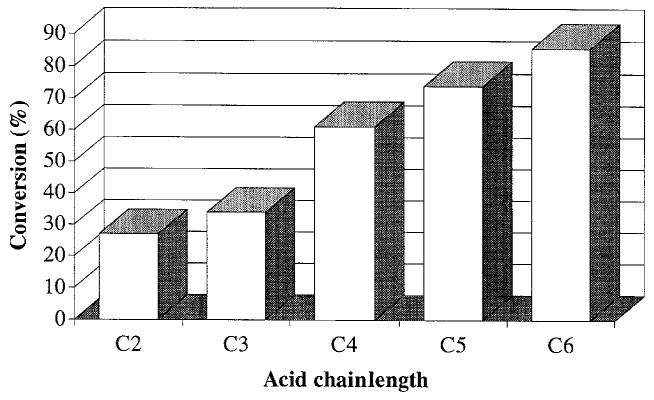

FIG. 6. Effect of acid chainlength on esterase 30,000 activity. Alcohol/acid molar ratio $R = 1{,}100$ mg of enzyme, at 37°C and 250 rpm.

Table 2
Effect of Alcohol Structure on Esterase 30,000 Activity[a]

Alcohol	Conversion yield (%)
Hexanol	86.4
2-Hexanol	22.6
cis-3-Hexenol	50
Geraniol	71.4
Nerol	75.9
Citronellol	85.8

[a]Alcohol/acid molar ratio of $R = 1{,}100$ mg of esterase 30,000, at 37°C and 250 rpm.

with geraniol and linear acids whose carbon chainlength varied between C_2 and C_6. The kinetics of enzymatic catalysis were determined under standard conditions. Figure 6 shows the variation of ester yield as a function of acid chainlength after 75 h. The conversion yields of geranyl acetate and geranyl propionate were low, about 30%. Beyond C_4, the conversion yield increased to 60% for geranyl butyrate, 74% for geranyl valerianate, and 85% for geranyl caproate. The activity of esterase 30,000 from *M. miehei* in organic solvent increases when the acid carbon chainlength increases, according to the study of Langrand *et al.* (27). Inhibition of the enzyme by short-chain acids can be due to the presence of acid functions in the water layer that moistens the enzyme, and the molar concentrations of these acid functions decrease when the acid carbon chainlength increases. However, another work (28) showed that the activity of lipase from *M. miehei,* immobilized on celite, decreased when the acid or alcohol carbon chainlength was increased. It seems that the activity of enzymes towards the substrates can depend on the enzymes' pretreatment (immobilization, chemical modification). It can also depend on the type of culture medium of the organisms from which the enzymes were extracted.

Effect of alcohol structure. To study the effect of alcohol molecular structure on the esterase 30,000 activity, esterification reactions of butyric acid catalyzed by esterase 30,000 were made with a primary saturated alcohol (1-hexanol), a secondary alcohol (2-hexanol), a primary unsaturated alcohol (*cis*-3-hexenol), and terpenic alcohols (nerol, citronellol). Table 2 shows that the conversion yield of ester obtained with the secondary alcohol (2-hexanol) is lower than that obtained with the corresponding primary alcohol (1-hexanol). This result is in accordance with classical chemical reactions and can be explained by the difference in reactivity of these two classes of alcohol. The high ester yield obtained with citronellol (86%), geraniol, and nerol (75%) contradicts the result obtained by Langrand *et al.* (27). They found a low activity for *M. miehei* lipase with terpenic alcohols. The low relative conversion yields obtained with unsaturated alcohol can be explained by low mobility of the alcohol groups. It seems that the isomer *cis/trans*, which exists between geraniol and nerol, has no effect on the catalytic activity of the enzyme.

Geranyl butyrate synthesis by other lipases. We tested the potential of other industrial lipases from various origins to synthesize geranyl butyrate in a solvent-free system (under standard conditions) and to hydrolyze olive oil. Table 3 shows conversion ester yield and hydrolytic activity obtained by each lipase. The best conversion yield and hydrolytic activity were obtained by *C. cylindracea* lipase, which were, respectively, 83.1% and 75.3 U/mg, followed by *P. fluoresense* lipase with an 80% conversion yield and 16.6 U/mg hydrolytic activity. The esterase 30,000 from *M. miehei* exhibited 71.4% conversion yield and 16.7 U/mg hydrolytic activity. The lipase from *C. cylindracea* had a hydrolytic activity five times higher than that of *P. fluoresense* and *M. miehei*. However, the conversion yields obtained by these lipases were of the same order. This shows that there is little correlation between the synthetic and hydrolytic activities, as reported by Wu *et al.* (29). They also reported that generally a high hydrolytic activity suggests a high synthetic activity, and the capability of industrial lipases to catalyze esterification reactions depends not only on the microorganisms from which the lipases came, but also on different batches and different producers. The result obtained with lipase from *R. javanicua*, which showed a low synthetic activity (4%) but a relatively high hydrolytic activity (15.5 U/mg), confirms that there is little correlation between lipases' synthetic and hydrolytic activities. Lipases from *R. niveus* and porcine pancreas gave the lowest synthetic and hydrolytic activities under our conditions.

TABLE 3
Geranyl Butyrate Synthesis and Olive Oil Hydrolysis by Other Enzymes[a]

Lipases	Origin	Protein (%)	Hydrolytic activity (U/mg of protein)	Conversion yield (%)
Rhizopus niveus	Fungus	41.3	6.0	3.4
Pseudomonas fluoresense	Bacteria	23.6	16.6	80
Candida cylindracea	Yeast	15.4	75 3	83.1
115 P of porcine pancreas	Mammal	59.8	2.6	11
R. javanicua	Fungus	41.6	15.5	4
Mucor miehei	Fungus	13	16.7	71.4

[a]Alcohol/acid molar ratio $R = 1.4$, 80 mg of lipase, at 37°C and 250 rpm.

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REFERENCES

1. Che Omar, I., N. Nishio, and S. Nagai, Fat Hydrolysis and Esterification by a Lipase from *Humicola lanuginosa, Agric. Biol. Chem. 51*:2153–2159 (1987).
2. Gandhi, N.N., S.B. Sawant, and J.B. Joshi, Studies on the Lipozyme-Catalysed Synthesis of Butyl Laurate, *Biotechnol. Bioeng. 46*:1–12 (1995).
3. Linko, Y.Y., M. Lämsä, A. Huhtala, and P. Linko, Lipase-Catalyzed Transesterification of Rapeseed Oil and 2-Ethyl-1-hexanol, *J. Am. Oil Chem. Soc. 71*:1411–1414 (1994).
4. Myrnes, B., H. Barstad, R.L. Olsen, and E.O. Elvevoll, Solvent-Free Enzymatic Glycerolysis of Marine Oils, *Ibid 72*:1339–1344 (1995).
5. Charlemagne, D., and M.D. Legoy, Enzymatic Synthesis of Polyglycerol-Fatty Acid Esters in a Solvent-Free System, *Ibid 72*:61–65 (1995).
6. Lämsä, M., A. Huhtala, Y.Y. Linko, and P. Linko, 2-Ethyl-1-hexanol Fatty Acid Esters from Rapeseed Oil by Transesterification, *Biotechnol. Technol. 8*:451–456 (1994).
7. Claon, P.A., and C.C. Akoh, Enzymatic Synthesis of Geranyl Acetate in *n*-Hexane with *Candida antartica* Lipases, *J. Am. Oil Chem. Soc. 71*:575–578 (1994).
8. Langrand, G., C. Triantaphylides, and J. Baratti, Lipase Catalysed Formation of Flavour Esters, *Biotechnol. Lett. 10*:549–554 (1988).
9. 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).
10. Talon, R., M.C. Montel, and J.L. Berdague, Production of Flavor Esters by Lipases of *Staphylococcus xylosus*, *Enzyme Microb. Technol. 19*:620–622 (1996).
11. Carta, G., J.L. Gainer, and A.H. Benton, Enzymatic Synthesis of Esters Using an Immobilized Lipase, *Biotechnol. Bioeng. 37*: 1004–1009 (1991).
12. 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).
13. Lima, F.V., 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).
14. Yee, L.N., C.C. Akoh, and R.S. Phillips, *Pseudomonas* sp. Lipase-Catalyzed Synthesis of Geranyl Esters by Transesterification, *J. Am. Oil Chem. Soc. 72*:1407–1408 (1995).
15. De Castro, H.F., P.C. de Oliveira, and E.B. Pereira, Evaluation of Different Approaches for Lipase Catalysed Synthesis of Citronellyl Acetate, *Biotechnol. Lett. 19*:229–232 (1997).
16. Bello, M., D. Thomas, and M.D. Legoy, Interesterification and Synthesis by *Candida cylindracea* Lipase in Microemulsions, *Biochem. Biophys. Res. Commun. 146*:361–367 (1987).
17. Macris, J.B., H. Stamatis, and F.N. Kolisis, Microemulsion as a Tool for the Regioselective Lipase-Catalysed Esterification of Aliphatic Diols, *Appl. Microbiol. Biotechnol. 46*:521–523 (1996).
18. Kim, S.M., and J.S. Rhee, Production of Medium–Chain Glycerides by Immobilized Lipase in a Solvent-free System, *J. Am. Oil Chem. Soc. 68*:499–503 (1991).
19. Ergan, F., M. Trani, and G. André, Use of Lipases in Multiphasic Systems Solely Composed of Substrates, *Ibid. 68*:412–417 (1991).
20. Tietz, N.W., and E.A. Fiereck, A Specific Method for Serum Lipase Determination, *Clin. Chim. Acta 13*:352–355 (1966).
21. Lowry, O.H., N.J. Rosebrough, R.A.L. Far, and R.J. Randall, Protein Measurements with the Folin Phenol Reagent, *J. Biol. Chem. 193*:265–275 (1951).
22. Karra-Chaabouni, M., S. Pulvin, D. Touraud, and D. Thomas, Enzymatic Synthesis of Geraniol Esters in a Solvent-Free System by Lipases, *Biotechnol. Lett. 18*:1083–1088 (1996).
23. 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 The Isoamyl Acetate, *Ibid. 16*:247–250 (1994).
24. 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).
25. Golberg, M., and M.D. Legoy, Water Activity as a Key Parameter of Synthesis Reactions: The Example of Lipase in Biphasic (Liquid/Solid) Media, *Enzyme Microb. Technol. 12*:976–981 (1990).
26. Zaks, A., and A.J. Russell, Enzymes in Organic Solvents: Properties and Applications, *J. Biotechnol. 8*:259–270 (1988).
27. Langrand, G., N. Rondot, C. Triantaphylides, and J. Baratti, Short Chain Esters Synthesis by Microbial Lipases, *Biotechnol. Lett. 12*:581–586 (1990).
28. Manjon, A., J.L. Iborra, and A. Arocas, Short-Chain Flavour Ester Synthesis by Immobilised Lipase in Organic Media, *Ibid. 13*:339–344 (1991).
29. Wu, X.Y., S. Jääskeläinen, and Y.-Y. Linko, An Investigation of Crude Lipases for Hydrolysis, Esterification, and Transesterification, *Enzyme Microb. Technol. 19*:226–231 (1996).

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