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Lipase-Catalyzed Production of Short-Chain Acids Terpenyl Esters of Interest to the Food Industry

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Abstract

The production of low molecular weight esters as flavor compounds by biotechnological processes has a potential interest for the food industry. The use of natural available substrates and enzymes is an essential part of the process design, because the products may obtain natural label. In this study, direct esterification of citronellol and geraniol with short-chain fatty acids catalyzed by free lipase from *Mucor miehei* was performed with high yields in *n*-hexane. The effects of the acid:alcohol ratio on the bioconversion rate of increasing chain length esters was investigated. To reach the optimum yield, substrates and enzyme concentration were determined. The inhibiting effects of acid are strongly attenuated by reducing the quantity of acid and increasing the amount of enzyme in media following the optimum values. Improvements have been made to increase the ester purity. The consumption of excess substrate by adding calculated amounts of acid gives a 10% yield enhancement, and leads to 100% pure terpenyl esters. The first steps to a scale-up application were attempted using a reactor that allowed us to produce ester quantities up to 100 cm^3. Separation and purification of the products were treated with success, underlining the lipase stability and efficiency under the conditions of this study. The ability to recover the enzyme, and reusing it in bioconversions, plays a major role in reducing the cost of the overall process.

Index Entries: Lipase; *Mucor miehei*; direct esterification; enzymatic synthesis; organic solvents; terpenyl esters.

Introduction

The production of low molecular weight esters as flavor compounds by biotechnological processes has a potential interest for the food industry.

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Such esters can be produced by an alcohol/acid mix reaction using either inorganic acids or enzymes as catalysts. The purpose of our work was to develop optimized ester syntheses, via enzymatic catalysis. This method allows molecules, considered as "natural products" in view of the regulations and requirements prevailing in the food industry, to be obtained. Enzyme-catalyzed reactions are superior to conventional chemical methods owing to mild reaction conditions, high catalytic efficiency, and inherent selectivity of natural catalysts, which result in much purer products. Concerning the chemistry of the reaction, the mechanism of ester-hydrolyzing enzymes is very similar to that describing chemical hydrolysis by a base. A nucleophilic group from the active site of the enzyme attacks the carbonyl group of the substrate ester. This nucleophilic "chemical operator" can be the hydroxyl of the serin group of the catalytic triad: asparagine, histidine, serine. The lipase-catalyzed hydrolyses were performed to hydrolyze esters other than glycerides, in the field of the organic synthesis. Lipases from *Mucor* species, such as *Mucor miehei*, have been used for biotransformations more recently. Such esterase reactions are frequently easily reversible, depending on the concentration of reactants or the availability of water. When used in organic solvents, lipases (triacyl glycerol hydrolases EC 3.1.1.3.) were described to catalyze not only ester hydrolysis, but also ester synthesis *(1–3)*. The amino acid sequence for the putative active site, with an essential serine residue, has been shown to be common to a range of protease, lipase, and esterase enzymes *(4,5)*. It was shown that a relatively nonpolar liquid phase can easily provide the necessary mass transfer, probably without greatly altering the composition, structure, or properties of the enzyme particles. The relationship between water content and enzyme activity has been an important topic of study in organic media. High water levels were shown to reduce the rate of lipase-catalyzed esterification *(6)*, presumably by causing hydrolysis of the acyl-enzyme intermediate. Study of the kinetics of lipase-catalyzed esterification, in organic media of varied concentrations of both acid and alcohol substrates, showed that hydration acts as a competitive inhibitor with the nucleophile alcohol, as expected for the acyl-enzyme mechanism, but also with the acid, probably because of competition for the binding site *(7)*.

Commercially available enzyme preparations were screened as a potential source for biotechnological production of short-chain terpenyl esters *(1,2,8–18)*. This approach has already been investigated for methyl acetate *(19)* and methyl propionate *(20)*, using *M. miehei* lipase. It has been shown to be nonspecific and efficient in the direct esterification of alcohols in solvent media. Previous studies *(1,2,13,17,18)* showed that it was difficult to prepare acetates by direct esterification. More recent studies *(15,21–24)* reported efficient preparation of propionates, butyrates, and caproates by direct esterification, transesterification, or interesterification, approaching molar conversion yields ranging from 90 to 100%. This work fully concerns the research and development of a performing bioconversion process for citronellyl and geranyl acetates, easily transposable to industrial scale, and

other production of terpenyl esters by direct esterification catalyzed by a lipase. The optimization of acetate synthesis is therefore an essential step for producing those esters by enzymatic synthesis. In the present study, we achieved the synthesis of citronellyl and geranyl acetate in *n*-hexane, with no additional water, catalyzed by *M. miehei* lipase. We optimized substrate and enzyme concentrations, solvent effects, kinetics, and biocatalyst reusability in order to design a rational process for future industrial application.

Materials and Methods

Materials

The commercial crude lipase powder esterase 30,000 from *M. miehei* was obtained from Gist-brocades (Seclin, France). The enzyme had a specific activity of 30 IU/mg and was used as received. Citronellol (95% pure) and geraniol (96% pure) were purchased from Fluka Chemika (Buchs, Switzerland). Acetic acid and all solvents were of analytical grade and obtained from Janssen. Natural ester bioprocess production involved natural certified substrates purchased from Daniel (Emmericer, Germany), SAF-ISIS (Soustons, France), and Néo Flaveur (Chevreuse, France).

Esterification Method

Ester synthesis was carried out in stoppered glass bottles (15 cm^3) as bioreactors, containing 10 cm^3 of *n*-hexane. No additional water was added to the reaction medium, as we considered that the water necessary to produce enzymatic activity is provided by the esterification reaction. All samples were prepared in duplicate and incubated at 37°C under magnetic stirring (280 rpm). For solvent selection trials, citronellyl and geranyl acetates were obtained using 0.250 mol/dm^3 of acid and alcohol, with 250 mg of lipase in a reaction medium containing 10 cm^3 of the tested solvent. Bioconversion rates were determined after a 5-h incubation period. The effects of substrate concentration were studied for different acid:alcohol molar ratios, in 10 cm^3 of *n*-hexane with 250 mg of lipase. The effects of enzyme concentration were studied on direct esterification of 0.25 mol/dm^3 geraniol and citronellol with 0.25 mol/dm^3 acetic acid, with 1–50 g/dm^3 of lipase preparation. The percentages of molar conversion were determined after a 24-h incubation period.

Analytical Method

After fixed reaction times, samples (0.5 µL) were withdrawn and analyzed by gas chromatography with a Varian 3300 Gas Chromatograph equipped with a flame ionization detector at 220°C and an on-column injector. The analyses were carried out on a fused silica capillary column CP Sil 5 CB (Chrompack, 25 m × 0.32 mm inner diameter, 5.00-µm film thickness), and operated isothermally at 220 or 250°C. Injector and detector temperatures were set at 270 and 300°C, respectively. Nitrogen was used as

Table 1
Influence of Solvent Nature on Lipase Activity

Solvent	Log *P* value[b]	Initial rate ($mmol \cdot dm^{-3} \cdot min^{-1} \cdot mg^{-1}$)[a] Citronellyl acetate	Geranyl acetate
Acetone	–0.23	0.024	0.014
Acetonitrile	–0.33	0.021	0.024
THF	0.49	0.023	0.029
Diethyl ether	0.85	0.030	0.108
Diisopropyl ether	1.7	0.036	0.129
Benzene	2	0.219	0.506
Toluene	2.5	0.526	0.776
Pentane	3	0.720	1.423
Hexane/ether (90/10)	3.235	0.406	0.333
Hexane	3.5	0.536	0.540
Heptane	4	0.271	0.953
Octane	4.5	0.194	0.426

[a]Coefficient of analysis was 1.75% and results were from two experiments.
[b]Source: *see* ref. *15*.

Initial rates of citronellyl and geranyl acetate synthesis are shown as a function of log *P* value. The reaction was carried out as defined under Materials and Methods, with 250 mg of lipase, 0.250 mol/dm^3 of acid, and alcohol.

the carrier gas at a total flow rate of 1.4 cm^3/min (split 400 cm^3/min). The extent of synthesis (percentage of bioconversion yield) was calculated on the basis of the amount of alcohol consumed in the reaction.

Results and Discussion

Effects of Solvent Nature on Lipase Activity

The hydrophobicity of organic solvents used in biocatalysis was shown to affect the enzyme activity *(25–28)*. The log *P* value of organic solvents is widely used to describe their hydrophobicity. Hydrophobic organic solvents (log $P > 4$) are believed to be well suited for biocatalysis *(26,29)*. However, there is no general trend of higher activity at higher log *P* values *(19,20)*. The influence of organic solvents on the esterification activity of *M. miehei* lipase was studied (Table 1). The enzyme showed some activity in all studied solvents. The reaction rate of citronellyl acetate and geranyl acetate synthesis was evaluated with selected solvents of increasing log *P* values of –0.23 to 4.5 as the continuous phase. Solvents of log *P* values of 2–4.5 were most supportive of terpenyl acetate synthesis. Pentane (log $P = 3$) shows the highest activity (0.72 $mmol \cdot dm^{-3} \cdot min^{-1} \cdot mg^{-1}$ enzyme for citronellyl acetate, and 1.14 $mmol \cdot dm^{-3} \cdot min^{-1} \cdot mg^{-1}$ enzyme for geranyl acetate). However, despite good performance, its high toxicity makes it unsuitable for the production of natural food flavors.

Contrary to the general trend, the initial enzymatic activity observed for citronellyl acetate biosynthesis was inversely proportional to the hydrophobicity of the organic solvents used (from pentane to octane). This can be related to the poor aqueous conditions (no additional water) of our reaction medium. Water provided by the first steps of the esterification reaction was not sufficient enough to allow high initial enzymatic activity. Furthermore, no preponderant influence of solvent hydrophobicity was observed.

Although more hydrophobic, heptane was more selective in the case of geranyl acetate, probably acting on lipase specificity control. Hexane shows lower activity, but better stability, in both citronellyl and geranyl acetate formation. The use of diethyl ether as cosolvent in hexane (90/10 [v/v]) to improve substrate solubility appeared to be ineffective, because the overall polarity of the bulk medium was less hydrophilic in this case than previously reported by us for methyl propionate synthesis *(20)*. The rates we observed in solvents with log $P < 2$ were lower; they were reported to disrupt the essential water layer around the enzyme *(16,18–20,30)*. The same trend was observed for both acetates. As we previously reported, the positive effects of minimizing the excess free water in the bulk reaction medium *(19,20)*, no water content optimization was attempted. *n*-Hexane will be used as a reactional solvent system, since it is compatible with EEC Legislation (88/344) relevant to solvent use in the food industry.

Synthesis of Citronellyl and Geranyl Acetates

Free lipase from *M. miehei* was tested for its ability to synthesize citronellyl and geranyl esters from acetic acid by direct esterification in *n*-hexane for various concentration ratios (Fig. 1). Even at the low concentrations used (ratio 0.5), citronellyl acetate and geranyl acetate reached 34.8 and 37.5% *M* conversion, respectively, which is in agreement with earlier findings *(1,2,8)*. A mean yield of 100% was reached for acetates after a reaction time of 24 h (results not shown), justifying the interest in the determination of optimal conditions as a rational step before the scale-up process.

Effects of Substrate Concentration

The effects of substrate concentration on the synthesis of flavor esters with *M. miehei* has been reported *(7,19–21,29,31,32)*. The effect of geraniol, citronellol and acetic acid concentrations on the esterification activities of *M. miehei* lipase are shown in Fig. 2A,B. The initial rates of esterification were found to depend on alcohol and acid concentrations. High initial rates were obtained for acetic acid <0.25 mol/dm^3. Increasing concentrations of acetic acid markedly inhibited *M. miehei*, leading to a 90% loss in lipase activity at concentrations of up to 0.3 mol/dm^3. Our results are consistent with acetic acid inhibition as reported by several studies *(1,8,10,22,33,34)*, since the rate was somewhat reduced at high acid:alcohol molar ratio. The very polar nature of acetic acid strongly favors its solvation in the aqueous phase.

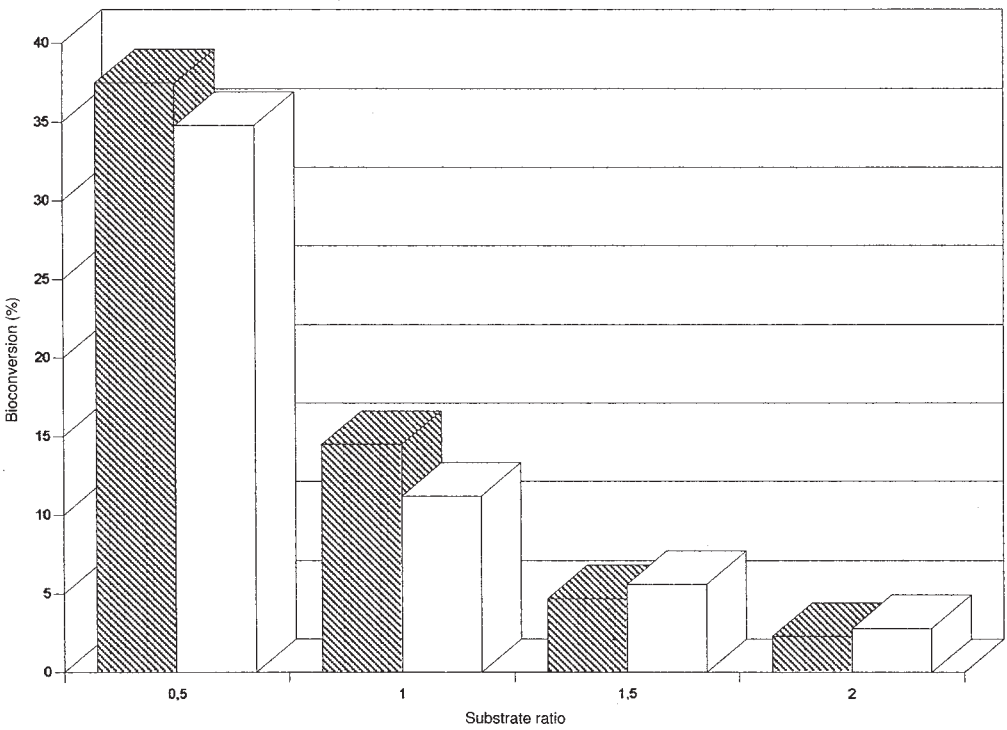

Fig. 1. Bioconversion yields of lipase-catalyzed synthesis of citronellyl acetate and geranyl acetate by direct esterification in *n*-hexane. The reaction mixtures were incubated with 250 mg of lipase for different acid:alcohol ratios. Samples were withdrawn after 5 h to determine conversion rates. (▨), geranyl acetate; (□), citronellyl acetate.

Consequently, the local pH decreases, and the enzyme active site is modified, making inhibitory interactions. Chulalaksananukul et al. *(26)*, working with lipozyme IM20 and IM60 from *M. miehei*, suggested that geraniol is an inhibitor of the lipase-catalyzed transesterification reaction. However, initial rates reported here were highly improved by increasing alcohol concentrations up to 1 mol/dm^3. Citronellol and geraniol had no inhibitory effect on the esterification activity, underlining the large efficiency of our catalyst.

Effects of Enzyme Concentration

The effects of varying enzyme concentration on the yield of terpenyl acetate production are given in Fig. 3. Direct esterification of geraniol and citronellol with acetic acid were investigated in a reaction volume of 10 cm^3 of *n*-hexane, for an acid:alcohol ratio of 0.5, and various enzyme concentrations. Molar conversion yield was determined after a 24-h incubation period. The rate of production of citronellyl and geranyl acetate increased with the amount of enzyme to reach 90 and 100% *M* conversion. The minimum concentration necessary to achieve a maximum yield was 25 g/dm^3 (54% [w/w] reactants). When dealing with geranyl acetate biosynthesis

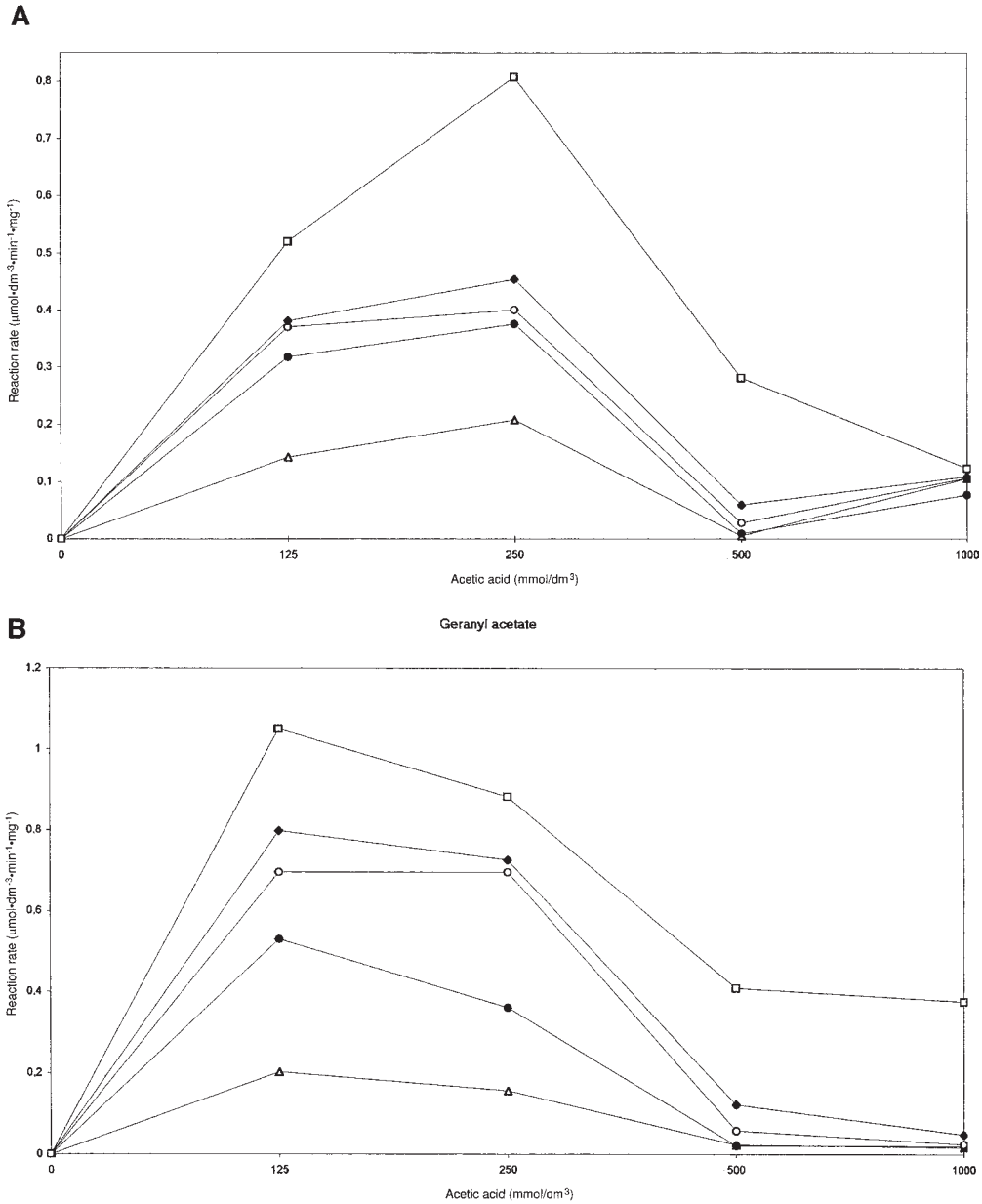

Fig. 2. Effect of acetic acid concentration on initial esterification rate of citronellol **(A)** and geraniol **(B)**, for various alcohol concentrations. (△), 0.125 mol/dm^3; (●), 0.250 mol/dm^3; (○), 0.375 mol/dm^3; (◆), 0.5 mol/dm^3; (□), 1 mol/dm^3. Each graph point represents the average of duplicate experiments.

catalyzed by *M. miehei* lipase, Langrand et al. *(2)* reported yields of approx 10 and 20% after 1 h of bioconversion with high enzyme concentration (93% [w/w] reactants). Chulalaksananukul et al. *(26)* and Langrand et al. *(1)* reported yields of 14 and 85% for geranyl acetate obtained by

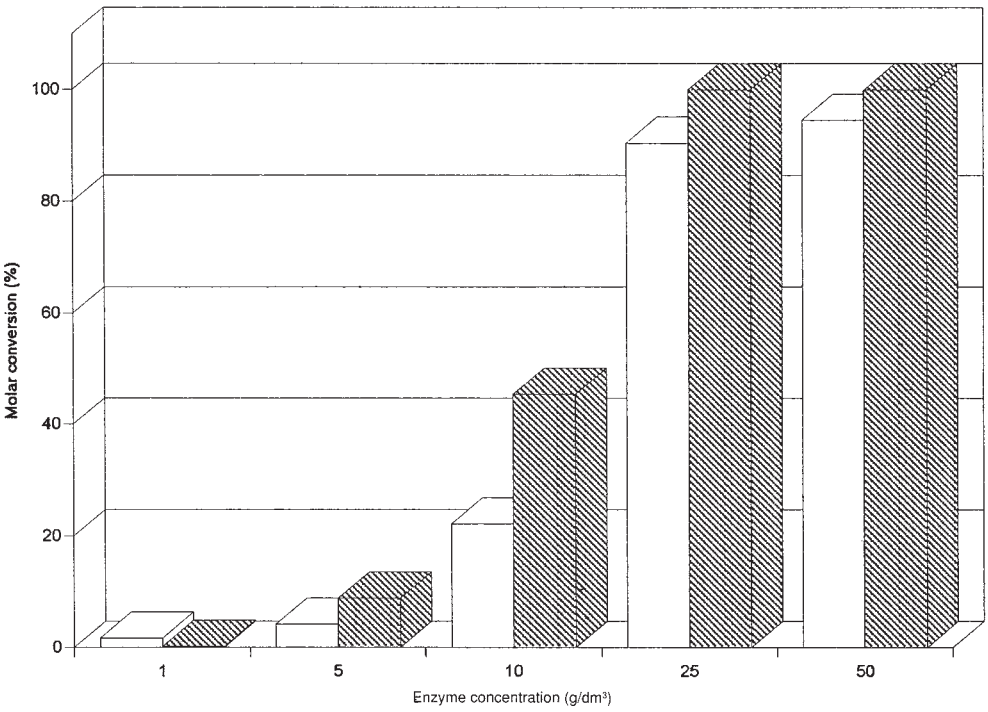

Fig. 3. Effect of enzyme concentration on direct esterification of geraniol and citronellol with acetic acid. Fixed acetic acid and alcohol concentration (0.250 mol/dm^3) were incubated with 1–50 g/dm^3 of *M. miehei* lipase. Molar conversion percentages were determined after 24 h of incubation. Each graph point represents the average of duplicate experiments. (□), citronellyl; (▨), geranyl.

transesterification reactions with enzyme concentrations of 12 and 37% ([w/w] reactants). The mass of enzyme preparation must be high enough to display the required level of catalytic sites and optimal diffusion of the reagents. Concentrations up to 50 g/dm^3 showed no efficient improvement in the biosynthetic activity. The substrate level in this case would be expected to have little impact on the phase behavior of the reactive mixture. However, increasing substrate concentrations relative to a fixed enzyme concentration ([w/w] reactants) resulted in a decrease in the bioconversion yield, in agreement with our previous results.

Kinetics of Acetates

In light of the preceding investigations, kinetics of lipase-catalyzed synthesis of citronellyl and geranyl acetates was determined (Fig. 4). By reducing the amount of acid, and increasing the amount of enzyme in media following the optimum values, the inhibiting effects of acetic acid was strongly reduced. The optimal conditions for lipase-catalyzed synthesis were 500 mg of enzyme, 0.125 mol/dm^3 of acid, and 0.25 mol/dm^3 of alcohol. After 4 h, geranyl acetate reached 92% conversion yield, whereas

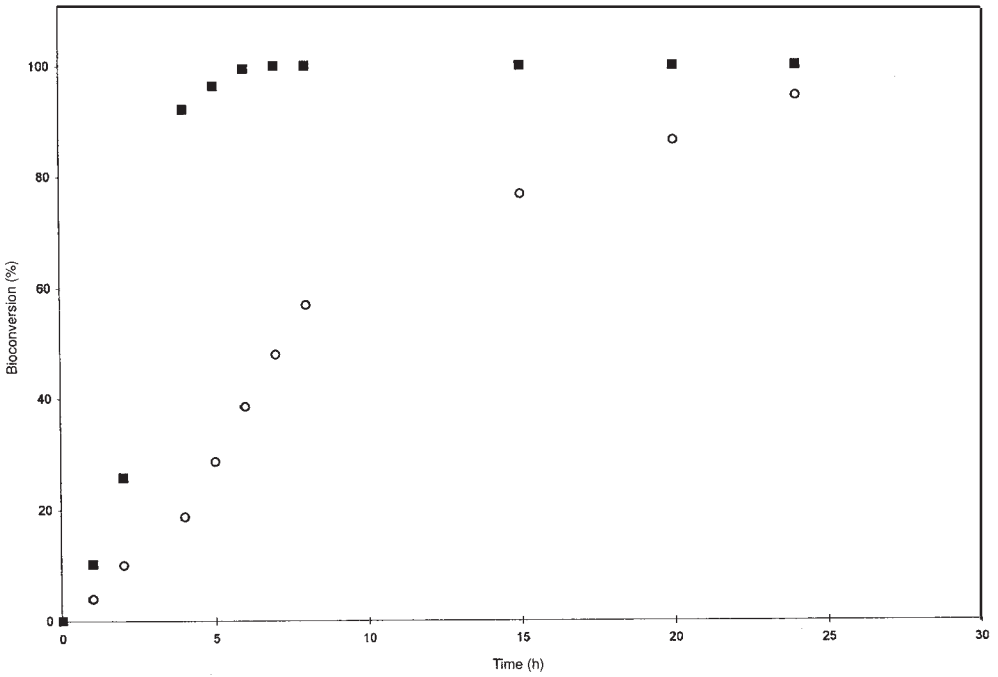

Fig. 4. Time course of enzymatic synthesis of citronellyl acetate (○) and geranyl acetate (■) in *n*-hexane under optimal experimental conditions. Samples were withdrawn at various incubation times and immediately analyzed. The results are the average of duplicate analyses.

citronellyl acetate needed 15 h to obtain 77% conversion. Optimal conversion yields (100%) were attained after 15 and 24 h of incubation, respectively. Direct esterification with lipozyme IM20 from *M. miehei*, reported by Langrand et al. *(1)*, yielded <0.5% geranyl acetate after 24 h, whereas Chulalaksananukul et al. *(26)* obtained 85% after 3 d of transesterification. In previous studies, Chulalaksananukul et al. *(25)* showed that geraniol excess led to an inhibition of the lipase-catalyzed transesterification reaction. Considering our reaction conditions, we noticed that the decreasing inhibitory effect of acetic acid was rather preponderant, whereas no inhibitory effect from geraniol was observed. Thus, the best reported yield of citronellyl acetate and geranyl acetate synthesis was, respectively, 98% conversion *(22)* and 100% conversion *(23)*.

Biosynthesis Process Setup

Enzyme and Solvent Recycling

During our study of free lipase catalysis, we noticed that the enzyme absorbed water generated by the reaction. Thus, lipase decantability becomes important when the reaction yield goes above 30%. While allowing an easy separation, the increase of lipase heaviness dramatically impedes the stirring conditions. Our previous work on natural methyl

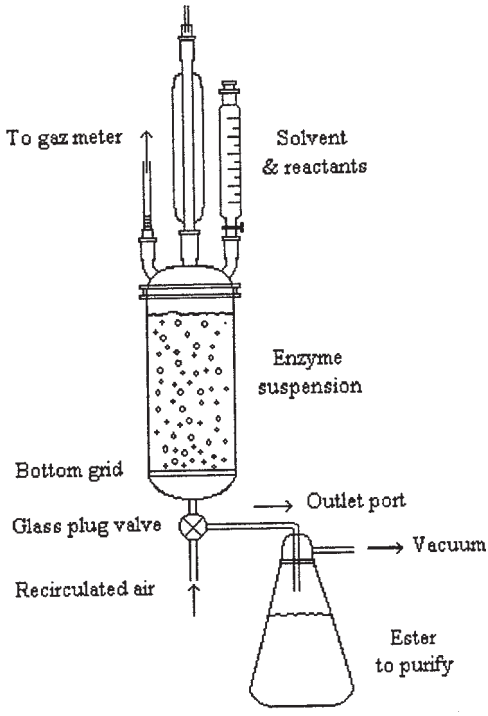

Fig. 5. Bioreactor process scheme.

propionate bioconversion *(20,35)* showed that *M. miehei* lipase was quite stable and could be reused repeatedly. To be used again, the enzyme must be dried in order to catalyze the reaction toward esterification. After acetone rinsing and low vacuum drying, lipase recovers its original activity in an organic medium. In our case, there was no need for enzyme fixation; free lipase separation was obtained by simple decantation and filtration, whereas its recycling became easier as the desorption risk disappeared. Depending on hexane utilization after reaction, the remaining ester and reagents can be preserved for a new reaction, or removed to perform purification by low-pressure distillation. Hexane is purified via a 1 mol/dm^3 sodium hydroxide solution extraction to hydrolyze or saponify ester traces. Thus, remaining alcohol, acid, and sodium alcoholates can be eliminated by washing with water. After distillation, followed by molecular sieve drying, hexane purity is better than 99%.

The reactor (Fig. 5) consisted of a 2-dm^3 operating volume column, with a glass grid of convenient porosity at the bottom. Lipase was suspended in *n*-hexane by means of air recirculated by a pump. Substrates in stoichiometric conditions were introduced by an additional funnel. After reaction, homogenization was stopped, and effluents were decanted by simple filtration through the bottom glass grid, operating under low vacuum pressure. Hydrated lipase was then suspended in an equal volume of acetone for dehydrating and dried in a vacuum following the procedure

previously described. Thus, lipase deterioration, solvent evaporation, and contamination risks were avoided.

Production of Natural Terpenyl Esters

Based on previous studies of optimization using acetates as a reaction model system, *M. miehei* lipase was tested for its ability to synthesize natural citronellyl and geranyl esters of propionic, butyric, and caproic acids by direct esterification in our bioreactor. To maintain efficient homogenization of the reaction system, the first experiments were carried out using 25 g/dm^3 of lipase concentration for various concentration ratios, in 2-dm^3 work-up volume. Figure 6 represents bioconversion yields observed after a 5-h reaction.

M. miehei lipase exhibited higher efficiency for increasing a fatty acid chain length, with an optimum with butyric and caproic acids. Terpenyl esters were converted with high yields using low acid concentrations. Propionate formation appeared to be stable with an increasing in the acid:alcohol ratio, although caproates showed an important reactivity for all tested concentrations. However, we noticed that excess acid concentration did not influence catalytic activity, justifying the uselessness to work at elevated acid:alcohol concentration ratios. Although equimolar ratios were shown to lower bioconversion kinetics, they allowed the reaction to be conducted at elevated concentrations. Considering a discontinuous bioprocess, it is more profitable to increase production rates and bioconversion time for the same production cycle. To optimize ester production yields, natural terpenyl ester biosynthesis by bioprocess was investigated for higher substrates and enzyme concentrations. Optimal production obtained by bioconversion in our triphasic fluidized-bed reactor is reported in Table 2 for various substrates and enzyme concentrations. By modeling lipase and substrate concentrations, it was possible to obtain high yields of natural terpenyl esters. Thus, production tests were successful because optimization of enzyme and substrate concentrations led to a 50% improvement compared to previous reported works. This allowed us to produce significant quantities of natural citronellyl and geranyl esters of short-chain acids, which are particularly difficult to synthesize and of interest to the food industry.

Conclusion

In many studies, specific lipases have been used to perform enzymatic synthesis of terpenyl acetates in organic media. Our results represent a significant contribution to the production of citronellyl and geranyl acetates by direct esterification with *M. miehei* lipase used in nonimmobilized conditions. With respect to all various works described in the literature concerning citronellyl or geranyl acetate formation, we developed in the present study one of the most efficient methods of biosynthesis. Good yields can be obtained (Table 2) by a rather simple way of processing (direct esterification, no additional water, easy solvent, and lipase recycling). We succeeded

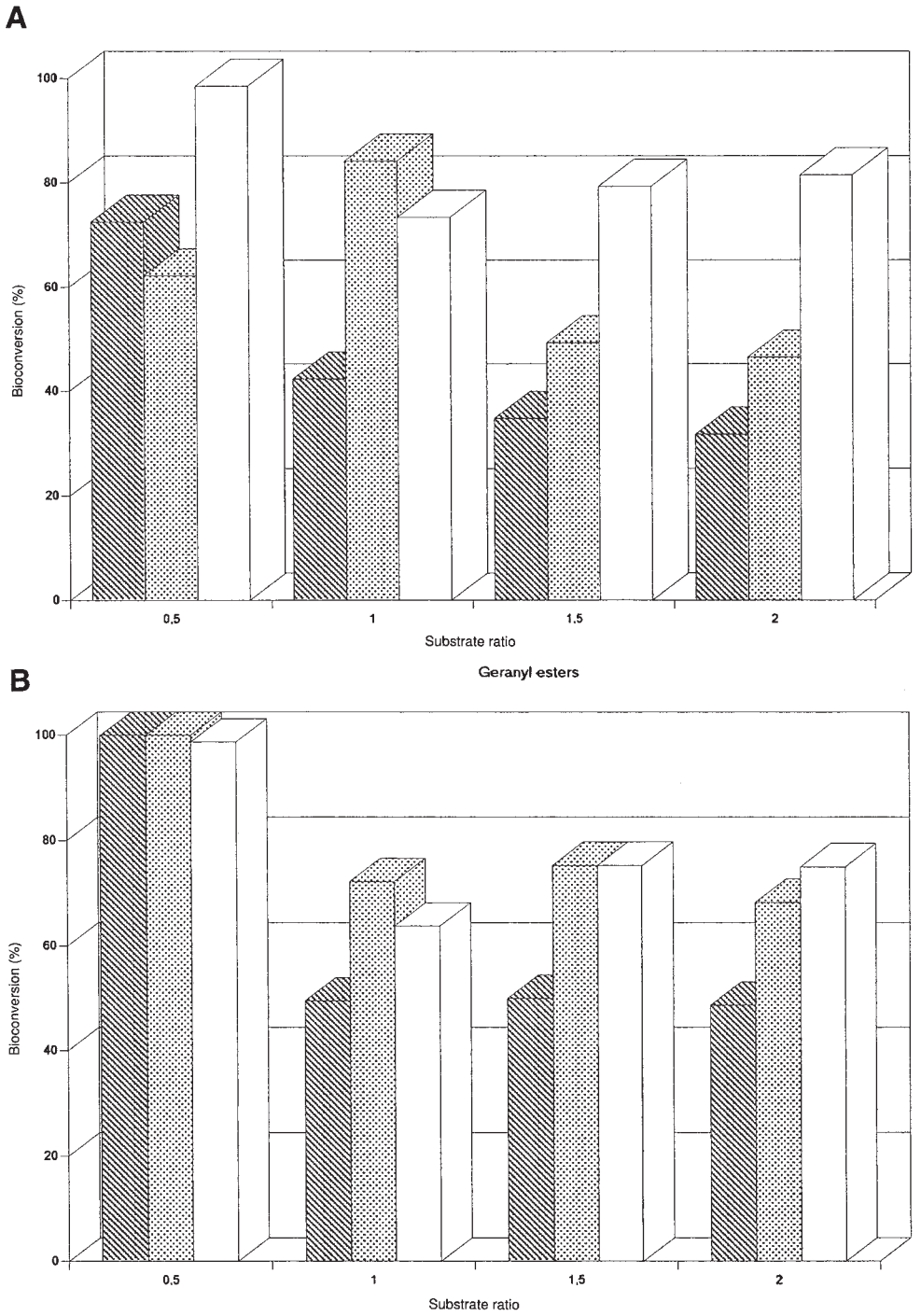

Fig. 6. Bioconversion yields of lipase-catalyzed synthesis of natural citronellol esters **(A)** and geraniol esters **(B)** by direct esterification in a triphasic fluidized-bed reactor. The reaction mixtures were incubated with 25 g/dm^3 lipase for different acid:alcohol ratios. Samples were withdrawn after 5 h to determine bioconversion rates. (▨), propionate; (⁞⁞), butyrate; (□), caproate.

Table 2
Optimal Production of Natural Terpenyl Esters
by Bioconversion in a Discontinuous Triphasic Fluidized Bed

Natural ester	Reaction yield (%)	Reaction time (h)	Concentration		Production g/dm³
			Substrates mol/dm³	Lipase g/dm³	
Citronellyl acetate	49	24	0.25	25	24
Geranyl acetate	100	15	$R = 0.5^a$	25	24
Citronellyl acetate	81	24	0.25	50	40
Geranyl acetate	100	6	$R = 0.5^a$	50	24
Citronellyl propionate	72	5	$R = 0.5^a$	25	19
Geranyl propionate	100	5	$R = 0.5^a$	25	26
Citronellyl propionate	98	4	0.25	50	48
Geranyl propionate	91	6	0.25	50	51
Citronellyl propionate	78	10	0.50	50	82
Geranyl propionate	88	10	0.50	50	92
Citronellyl butyrate	84	5	0.25	25	47
Geranyl butyrate	100	5	$R = 0.5^a$	25	28
Citronellyl butyrate	87	10	0.50	50	98
Geranyl butyrate	95	10	0.50	50	106
Citronellyl caproate	98	5	$R = 0.5^a$	25	31
Geranyl caproate	99	5	$R = 0.5^a$	25	31
Citronellyl caproate	70	10	0.50	50	89
Geranyl caproate	80	10	0.50	50	100

[a][Acid]:[alcohol] ratio of 0.125 mol/dm³ acetic acid for 0.25 mol/dm³ citronellol or geraniol.

in minimizing the acetic acid inhibitory effect on enzymatic activity, using fixed concentrations, lower than 0.25 mol/dm³. Thus, we determined the optimal conditions for 500 mg of the powdered enzyme as follows: 0.125 mol/(dm³·acid) and 0.25 mol/(dm³·alcohol). Under the conditions of the present study, the enzyme exhibited a broad substrate specificity, together with a high degree of stability and efficiency.

We extended successfully our biosynthetic method to elaborate other terpenic esters, with satisfying bioconversion results (Table 2). The free biocatalyst appears to be quite stable and can be reused repeatedly, even after undergoing washes with acetone and drying. The first steps to a scale-up application were attempts to develop a discontinued triphasic fluidized-bed reactor. Although this kind of process has a lower theoretical yield than a continuous one, it performs easy liquid/solid separation and enzyme recycling, lowering production costs. Although still under development, this reactor should produce more than 100 g of natural terpenyl esters a day, satisfying the annual consumption of our industrial partner in only a week. Successful extension of the production process to other natural flavoring esters was achieved by controlling different production steps, underlining the potential efficiency of the overall process for industrial-scale application.

Acknowledgments

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