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Enzymatic synthesis of geraniol esters in a solvent-free system by lipases

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Abstract

Geraniol esters were synthesised by direct esterification catalysed by esterases and lipases (five enzymes were tested) in a solvent-free system at 37 °C. The best conversions yields, about 85 %, on geranyl butyrate and valerate obtained with esterase 30 000 from *Mucor miehei*. The effect of substrate molar ratio alcohol/acid variation was studied. A study of the water production was made in parallel during the esterification reaction.

Introduction

Terpenoid alcohol esters are organic compounds that present a great interest in the food, cosmetic and pharmaceutical industries. Usually these esters are obtained by traditional methods such as chemical synthesis or extraction from natural sources. With the great interest given to natural products, the manufacturers were more interest by the use of the biotechnology to produce the natural aromas, in particular with enzymatic engineering.

During the last years, several works were carried out to synthesize terpenoid alcohol esters in particular geraniol esters by direct esterification in organic medium catalysed by lipases (Langrand *et al.*,1988, 1990; Nishio *et al.*, 1987; Claon *et al.*,1993, 1994).

To avoid problems of separation, toxicity and flammability of organic solvents, results have sought to eliminate the organic solvents and use a biphasic system with two phases liquid-solid containing the reactants in the liquid phase and the enzymatic preparation in the solid phase, this approach was used to synthesize sugar acetal fatty acid esters (Fregapane *et al.*,1991), butyl laurate (Gandhi *et al.*,1995), n-butyl oleate (Leltgeb and Knez ,1990), medium-chain acyl-glycerol (Kim and Rhee,1991) and recently to produce the geraniol esters by *Candida antarctica* immobilised lipase (Oguntimein *et al.*,1995).

In this work, we report the use of different industrial preparations of lipase to obtain geraniol esters in a solvent-free system (non addition of any external solvent) .

Materials and methods

Materials : Esterases from *Mucor miehei* and lipase from *Rhizopus arrhizus* were used in a powder form, supplied by Gist-Brocades (Seclin, France), the activity of each enzyme is expressed in GBE unit (glycerol butyrate esterase) based in the enzymatic hydrolysis of glycerol tributyrate emulsion at pH = 7.5 and at 40 °C :
- *Mucor miehei* : esterase 30 000 (30 000U/g), Piccantase B (400u/g), lipase 193 (26 000U/g).
- *Rhizopus arrhizus* : Lipase 5700 (154 000 U/g).

We used Lipozyme™, lipase derived from *Mucor miehei* and provided by Novo Nordisk, this enzyme was immobilised onto a macro porous anion-exchange resin of particle size 250-500 µm, its activity is 25 BIU/g (BIU = batch interesterification unit, defined as micro moles of palmitic acid incorporated into triolein per minute at 40°C). Geraniol, butyric and valeric acids were purchased from Fluka Chemie (France) with respective purities 99.5 %, 96 % and 98 %. Propionic acid was purchased from Labosi.

Esterification : the esterification was carried out in capped tubes, the reaction mixture consisting of 1 g, of alcohol and acid taken at 1 : 1 molar ratio and 0.1 g of crude enzyme, the tubes were incubated in orbital shaker where the temperature was fixed at 37 °C, and agitation speed at 250 rev/ min, a control without enzyme was incubated in parallel.

At accurate interval of time, samples of 20 microliters were taken, diluted in 1 ml of hexane which contain 10 microliters of hexanol as internal standard, and analysed by CG.

Analyses : the reaction was followed by measuring the quantity of departed acid by gas chromatography Carlo Erba equipped by a flame ionised detector (FID), the used column was HP-20M (carbowax) 25 x 0.32 x 0.3 mm thickness (USA). The oven temperature was maintained at 50 °C and then increased to 150 °C at a rate of 10 °C/min. Injector and detector temperatures were fixed at 250 °C. The water quantity was determined by coulometric measuring with microprocessor tetrator type aquaprocessor according the Karl Fisher and use the hydranal-coulomat AD reactive.

Results and discussion

Synthesis of geranyl butyrate by esterase 30 000

A study of synthesis of geranyl butyrate by esterase 30 000 was made, at first we investigated the effect of the amount of enzyme on the synthesis, the reaction mixture consist of 1 g of substrates taken at 1 : 1 molar ratio, the quantity of enzyme was varied, the concentrations used were 0.02, 0.05, 0.1 and 0.15 g., the esterification was carried out at 37 °C and the agitation speed was 250 rev/min. The table 1 shows the conversion yield variation as function of enzyme concentration, the conversion yield increase if the enzyme concentration increase, for ratio alcohol/acid = 1 and 0.1 g of enzyme, it can be seen that the synthesis yield reaches 85 %.

A high yield of conversion in geranyl butyrate was obtained in a solvent-free system by Oguntimen *et al.* (1995) at 60 °C and by the use of an immobilised enzyme from Novo : the Novozym 425™ of *Candida antarctica*.

% enzyme	Initial rate in mmoles/hours	Yield (% mol/mol)
2	0. 011	17
5	0.021	30
10	0.118	85
15	0.202	88

Table 1 : Effect of enzyme concentrations (% w/w) in the initial rate of geranyl butyrate synthesis

We also studied the effect of substrate molar ratio variation, the esterification was made on the same conditions as described previously, but the substrate molar ratio (alcohol/acid) was varied and it taken at 0.2, 0.5, 1, 2 and 5, the enzyme amount was 0.1 g.

The figure 1 shows the effect of molar ratio alcohol/acid variation, we noted that the conversion yield decrease with the decrease of the ration alcohol/acid, consequently the butyric acid has an inhibitor effect on the enzyme, probably by acidification of the aqueous medium which surrounds the enzyme as described by Welsh and Williams (1990).

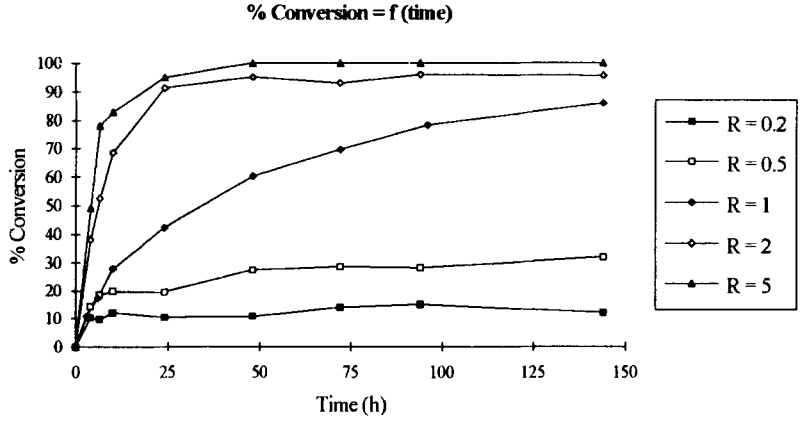

Figure 1 : Effect of substrate molar ratio alcohol/acid (R) on the geranyl butyrate synthesis catalysed by the esterase 30 000

In the esterification reactions, the water constitute an important factor to study, because it takes place at two levels, in one hand, it has an effect on the thermodynamic balance of chemical reaction, since this one is reversible, the water favours the hydrolysis and inhibits the esterification, on the other hand the water activates the enzyme and permits a good functioning of catalysis (Zaks and Russel, 1988; Jongejon *et al.*,1994). The production of water as a function of time was followed by measuring the amount of water formed by Karl Fisher method, the fig. 2 shows an increase of the water quantity with the time, we found that the quantity of water measured corresponds to the quantity of ester synthesised.

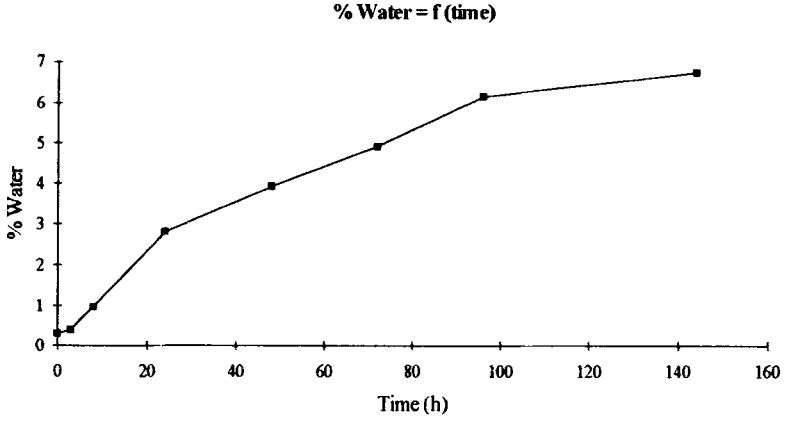

Figure 2 : Percent of water formed during of the geranyl butyrate synthesis catalysed by esterase 30 000

In order to investigate the effect of water on the enzyme activity , 1 % of water was added to the reaction mixture, at the end of the reaction , the conversion yield does not exceed 50 % (data no shown), consequently the enzyme activity decrease. This is comparable with the result found by Sudhir and all (Sudhir *et al.*,1995) who studied the effect of different amounts of water added to the reaction mixture (non-aqueous system) on the esterification of geraniol of palmarosa oil with n-butyric acid using immobilised lipase from *Mucor miehei.* This study shows that as the water increased the conversion rates decreased sharply, due to the low solubility of geraniol in the water which reduces the contact of the geraniol with the enzyme. Another study of the effect of water on the enzymatic esterification of n-butanol with the butyric acid in organic medium was made by Monot *et al.*(1991), they found that at low water concentrations (1g/l-5g/l), the initial rate increased up to a maximum then decrease to a minimum at higher water concentrations (10g/l), at a water concentration of 25 g/l, a biphasic system (water-organic solvent) was formed, in this case the initial esterification rate was restored and the reaction esterification was complete.

Synthesis of butyrate geranyl by different lipases

Two others *Mucor miehei* enzymes and a *Rhizopus arrhizus* lipase provided by Gist-Brocades were used to catalyse the synthesis of geranyl butyrate, the reaction was carried out at the standard conditions (see materials and methods). The results obtained are presented by the table 2 which shows the conversion rates on ester after 96 hours. A high yield was obtained with the esterase 30 000 (85 %), followed by the Piccantase B (80 %), then the esterase 193 (63 %), however the yield obtained with the lipase 5 700 of *Rhizopus arrhizus* is low (< 10 %). These results confirm that the *Mucor miehei* lipases are good catalysts for the formation of geranyl butyrate in a solvent-free system.

Immobilised enzymes have been frequently used during the last years, in particular for the production of flavour esters in organic media (Gillies *et al.*,1987;Carta *et al.*,1991; Manjon *et al.*,1991;Vazquez *et al.*,1994). In this work, we use an immobilised enzyme of *Mucor miehei,* the Lipozyme, in this case the initial rate is important, the yield reaches 66 % at 96 hours. In order to test the reuse of the Lipozyme, the reaction mixture was removed, the enzyme washed with the n-hexane, dried and placed in a new reaction mixture, the enzyme lost all activity as soon as the first reusability, this result is in according to the observation of Oguntimein et *al* (1995).

Enzymes	initial rate in mmoles/hours	conversion rates (%)
Esterase 30 000	0.118	85
Piccantase B	0.084	80
Esterase 193	0.080	63
Lipozyme	0.238	66
Lipase 5700	ND	<10

Table 2: Effect of different enzymes on the geranyl butyrate synthesis

Synthesis of geranyl propionate and geranyl valerate by esterase 30 000

The esterification of geranyl propionate and geranyl valerate was carried out in capped tubes, the reaction mixture consisting of 1 g of geraniol and propionic acid or valeric acid taken at 1 : 1 molar ratio and 0.1 g of crude enzyme (esterase 30 000).

After 96 hours of reaction incubation, the conversion rate on the geranyl valerate was about 85 %, this rate of esterification is comparable to that obtained with butyric acid (fig. 3). However the initial rate is more important, at 48 hours, it reaches a plateau.

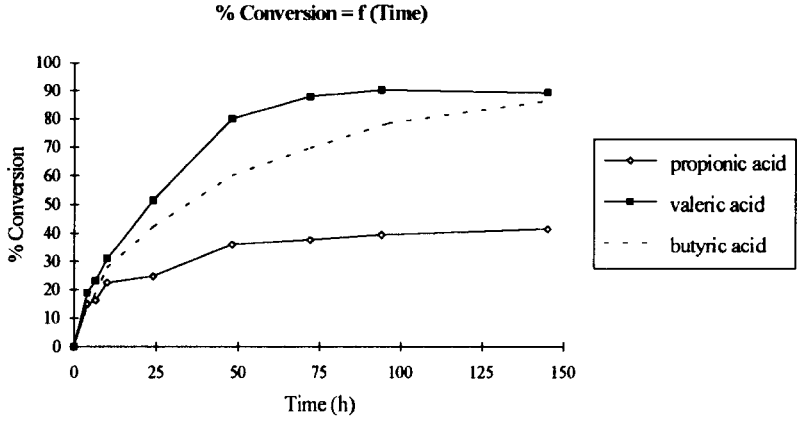

Figure 3 : Effect of Variation chain length of acids
(propionic, valeric and butyric acids) on the esterase 30 000 kinetics

The fig 3 shows that the initial rate of the synthesis of geranyl propionate is as important as those of geranyl butyrate and valerate, then the enzyme is inhibited and the conversion rate does not exceed 40%. Similar results have been reported by Langrand *et al.*(1990) by the investigation of the influence of the acid chain length on ester synthesis by different lipases, they found that *Mucor miehei* lipases were more active with long chain acid (C4-C6).

Conclusion

Terpenoid alcohol esters are important components of fragrances. Several works contribute to synthesis esters by lipases in different systems such as micro aqueous (system consisting of enzyme and organic solvent), biphasic (system consisting of water and organic solvent) and micellar (system is visually homogeneous consisting of water phase and organic solvent phase separated by a surfactant layer) (Borzeix *et al.*,1992), we note the use of organic solvent to dilute the substrates in order to eliminate the inhibition of enzyme by the excess of substrate, the reaction mixture concentrations are generally from 0.1 to 0.25 mole/l.

In this work we synthesised geraniol esters having the seal "natural", by esterification reactions catalysed by lipases in a solvent-free system. As a result of this study, we can present the following observations :

- *Mucor miehei* esterase (powder form) and lipase (Lipozyme immobilised) can function in a solvent-free

system in presence of high concentrations of substrates (about 4 moles/l), consequently the product concentration is higher in a solvent-free system than in organic solvent (at least ten times).

- The conversion rate on geranyl butyrate catalysed by *Rhizopus arrhizus* lipase 5700 is < 10 %, however lipases from *Rhizopus arrhizus* are among the predominant ones used for ester synthesis in organic solvents, therefore they can't function in high substrate concentrations.
- The quantity of water produced during the esterification increases as function of time and corresponds to the quantity of ester synthesised.
- The addition of 1 % of water (w/w) to the reaction mixture decrease the conversion rate.
- Esterase 30 000 activity is affected by the acid chain length, it is more efficient with butyric and valeric acids than with propionic acid.

References

Borzeix, F., Monot, F. and Vandecasteele, J. P. (1992). Enzyme Microb. Technol. 14, 791-797.
Carta, G., Gainer, J. L. , and Benton, A. H. (1991). Biotechnol. Bioeng. 37, 1004-1009.
Claon, P. A. and Akoh, C. C. (1994). J. Am. Oil Chem. So. 71, 575-578.
Claon, P. A. and Akoh, C. C. (1993). Biotechnol. Lett.15, 1211-1216.
Fregapane, G., Sarney, D.B. and Vulfson, E. N. (1991). Enzyme Microb.Technol. 13, 796-800.
Gillies, B.,Yamazaki, H. and Armstrong, D. W.(1987). Biotechnol. Lett. 9, 709-714.
Gandhi, N. N., Sawant, S. B. , and Josh,i J. B. (1995). Biotechnol. Bioeng. 46, 1-12.
Jongejon,J. A., Vantol ,J. Bert, A., Duine, J. A. (1994). Chimicaoggi/chemistry today, 15-24.
Kim, S. M. and Rhee, J. S. (1991). J. Am. Oil Chem. So. 68, 499-503.
Leltgeb, M. and Knez, Z. (1990). J. Am. Oil Chem. So. 67, 775-778.
Langrand, G., Rondo,t N., Triantaphylides, C. and Baratti, J.(1990). Biotechnol. Lett. 12, 581-586.
Langrand, G., Triantaphylides, C. and Baratti, J. (1988); Biotechnol. Lett. 10, 549-554.
Manjon, A., Iborra, J. L. and Arocas, A. (1991). Biotechnol. Lett. 13, 339-344.
Monot, F. , Borzeix, F., Bardin, M., and Vandecasteele, J. P. (1991) Appl. Microbiol. Biotechnol 35, 159-165.
Mestri, S. D. and Pai, J. S. , (1995). Biotechnol. Lett. 17, 459-461.
Nishio, T., Takahashi, K., Yoshimoto, T., Kodera, Y., Saito, Y.and Inada, Y.(1987). Biotechnol. Lett. 9, 187-190.
Oguntimein, G. B., Anderson, W. A., Moo-Young, M. (1995).Biotechnol. Lett. 17, 77-82.
Valivety, R. H., Halling, P. J. and Macrae, A. R. (1993). Biotechnol. Lett. 15, 1133-1138.
Vazquez, L. F., Pyle, D. L. and Asenjo, J. A.(1994). Biotechnol. Bioeng., 46, 69-79.
Welsh, F. W. and Williams, R. E. (1990). Enzyme Microb. Technol. 12, 743-748.
Zaks, A. and Russel, A. J. (1988). J. Biotechnol., 8, 259-270.