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Article

Production of Terpene Ester by Lipase in Non-Conventional Media

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Uma lipase imobilizada, preparada comercialmente, foi utilizada para catalisar a reação de esterificação do citronelol com o ácido butírico em n-heptano. Foi realizado uma série de experimentos para determinar a influência de diversos parâmetros na formação do butirato de citronila por esta preparação enzimática. Taxas de esterificação maiores que 95% foram obtidas nos processos efetuados em regime de batelada a 60 °C. A reação de síntese foi favorecida pela manutenção de baixos teores de água do meio reacional. A limitação das concentrações de ácido butírico ou citronelol resultaram no aumento do teor de água no meio reacional. Em condições aproximadas de equimolaridade, a água formada como sub-produto foi adsorvida pela fase sólida da enzima. Isto permitiu manter quantidades restritas de água no meio reacional, favorecendo a reação no sentido direto (reação de esterificação).

An immobilized commercially-prepared lipase was used to catalyze the esterification reaction of citronellol and butyric acid in n-heptane. A set of experiments was carried out to verify the influence of various parameters on the formation of citronellyl butyrate by this lipase. Esterification conversion yields above 95% were obtained under batch conditions at 60 °C. The equilibrium between the forward and reverse reactions was controlled by the level of water in the reactor vessel, which was built up when the synthesis was limited either by the butyric acid or citronellol contents. In the presence of nearly equimolar amounts of the reactants, the water formed as a by-product was found to be adsorbed by the solid enzyme phase. This allowed for the maintenance of restricted amounts of water in the reaction in the desired direction.

Keywords: *lipase, esterification, citronellyl butyrate*

Introduction

Terpene esters are very important aroma compounds. Currently, most flavor components are provided by traditional methods which include chemical synthesis or extraction from natural sources[1,2]. With the demand for natural products, the flavor industry is interested in the use of biotechnology to produce natural flavors[3]. In this context, it is not surprising that many research efforts have been dedicated to the use of lipases for the production of flavor esters[4-7].

The ability of lipase to catalyze synthetic reactions came under investigation in the 70's[8-11]. However, only recently have the difficulties associated with these kind of reactions apparently been solved by combining knowledge and skillful applications of modern experimental techniques, such as the use of biocatalysts in organic media[12,13]. High performance esterification reactions have been achieved by using either immobilized or free lipase preparations[14,15]. The application of immobilized biocatalysts in organic media has additional advantages over those already demonstrated in aqueous media. They keep the enzyme in a homogeneous dispersion and fix the water around it, in this way maintaining its native three-dimensional structure[16,17]. Such additional features are essential for the enzyme to be active in non-aqueous solutions[18].

For a process development in non-aqueous media, as a consequence of these considerations, we chose to focus our

work on the application of an immobilized biocatalyst, namely, lipase preparations, to perform esterification reactions on terpene alcohol as a starting compound[19-21].

As an example, we have selected the esterification of (*R/S*) citronellol with butyric acid, since this is a well documented system[22-26], and the results in the literature can provide a basis for comparison with the present study. The enzyme employed was a commercial preparation supplied by Novo Nordisk (Lipozyme IM[20]), which is a fungal lipase from *Mucor miehi* immobilized on macroporous synthetic resin[27]. The special properties of this immobilized preparation, such as high activity and stability at temperatures up to 60 °C and the ability to retain its essential water using solvent-free or organic media, may explain its widespread use in laboratory investigations[28-32]. Nevertheless, esterification reaction performance is still associated with many difficulties, and several parameters which affect the enzymatic synthesis in an organic solvent have yet to be elucidated. This work was carried out to get a better understanding of how different conditions influence the terpene ester production by using a particular immobilized lipase preparation. Special attention was given to the control of the water content in the bulk media without any addition of the desiccated agent.

Experimental

The enzyme, Lipozyme IM[20] (24 BIU/g) was kindly donated by Novo Nordisk (Denmark), and used as supplied (10% moisture content). The reactants (R, S citronellol, approx. 95%, and butyric acid, approx. 99%) were dehydrated before use, with 0.32 cm molecular sieves (aluminum sodium silicate, type 13 X- BHD Chemicals, Canada). Dry *n*-heptane, dried over molecular sieves, was used as the solvent for all experiments. All of the reagents used were purchased from Sigma Chemical Co., USA. Batch runs were carried out in capped 100 mL glass vials, with reciprocating agitation (150 rpm). The temperature was kept at either 40 or 60 °C. The substrate consisted of 0.30 M of butyric acid and 0.25 M of citronellol, except when the molar ratio was an experimental variable. In the latter tests, the following molar ratios (BA: CITRO) were used: 0.3, 0.6, 1.2, 2.5 and 4.0. The level of Lipozyme used was 25 mg/mL of substrate, except where shown in the text. Water concentrations in the liquid phase were determined by using a coulometric Karl Fischer Titrator (Mitsubishi Moisture Meter Model CA-06, Mandel Scientific Co. Ltd., Canada). Dry weight measurements of Lipozyme were performed gravimetrically, after drying the samples at room temperature for 60 min, followed by drying overnight at 100 °C. The reactions were monitored by measuring citronellol and butyric acid concentrations on a Shimadzu 9A gas chromatograph (Tekscience, Canada) with FID, using a GP 5% DEGS-PS column at 140 °C (Supelco, Canada), and linalool as an internal standard. The results were evaluated by calculating the citronellol conversion rates, as follows:

$$\text{Conversion rate (\%)} = \frac{Ci - Co}{Co} \times 100 \quad (1)$$

where: Ci is the initial concentration of citronellol, and Co is the concentration of citronellol at a given time.

Results

Influence of the Lipozyme content on the reaction kinetics

Esterification reactions were carried out at 40 °C with reciprocal agitation, by using butyric acid and citronellol at a molar ratio of 1:1.2, and enzyme concentrations in the range of 2.5 to 25 mg/mL. The completion of the reaction was very dependent on the enzyme concentration (Fig. 1). Conversion rates over 90% were achieved in 24 h when high enzyme concentrations were used (25 and 12.5 mg/mL). Reactions carried out with lower enzyme concentrations (5 and 2.5 mg/mL) required more than 100 h of incubation in order to attain conversion rates in the range of 70-80%.

Such performance could be improved by increasing the incubation temperature up to 60 °C, as recommended by the manufacturers[27]. As shown in Fig. 2, the time required with higher enzyme concentrations decreased to less than half when the temperature of incubation increased from 40 to 60 °C. Another interesting observation in this set of experiments was related to the concentration of water in the

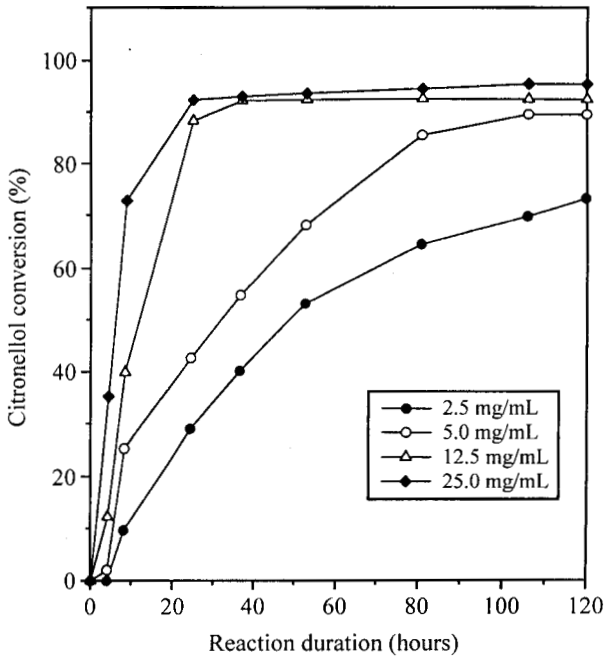

Figure 1. Effects of Lipozyme content on the citronellol conversion rate (%) as a function of time. A molar ratio between butyric acid and citronellol of 1.2:1 was used at 40 °C for Lipozyme contents of 2.5, 5.0, 12.5, and 25.0 mg/mL.

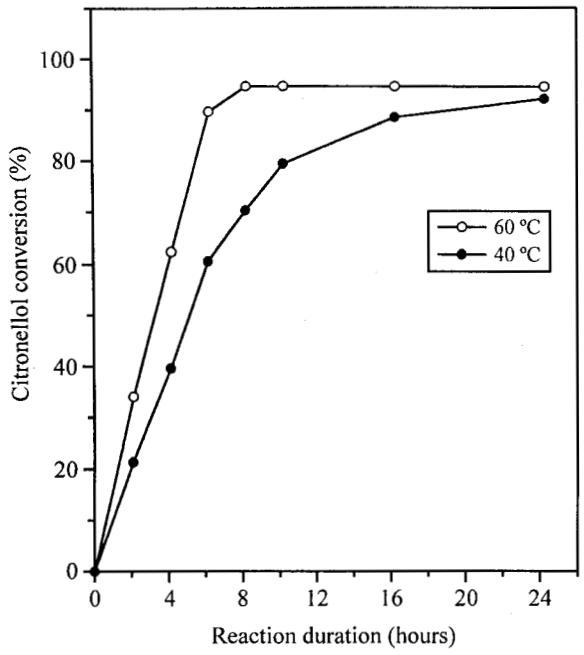

Figure 2. Effects of incubation temperature on the citronellol conversion rate.

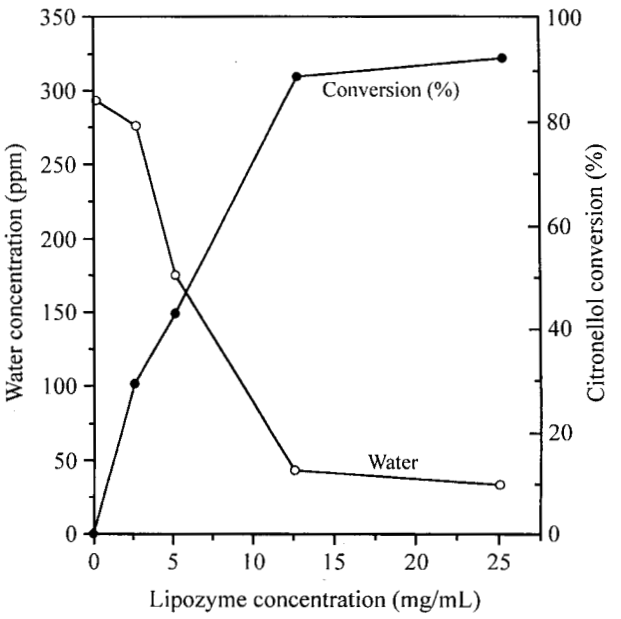

Figure 3. Relationship between the citronellol conversion rate and the water content in the reaction media after 24 h of reaction.

liquid phase, which was found to decrease as the enzyme concentration was increased (Table 1). The water content was as low as 30 mg/L for higher enzyme concentrations. For lower enzyme concentrations (2.5 mg/mL and 5.0 mg/mL), the water concentration in the liquid phase decreased slightly during the first 24 h of incubation, and then reached a constant value of 150-250 mg/mL. This effect could be attributed to the high capacity of Lipozyme to bind water, acting as a partial water buffer for the whole system. Therefore, the Lipozyme content played not only a kinetic role, but acted as a receptor for water during the reaction as well. In this context, a suitable proportion of Lipozyme should be used to bind all water generated during the reaction. So, under the conditions used in this set of experiments, enzyme concentrations higher than 15 mg/mL ensured optimal water content for performing the esterification reaction (Fig. 3).

Table 1. Monitoring the water content (ppm) in the liquid phase as a function of time for substrates containing different amounts of Lipozyme.

Lipozyme content (mg/mL)	Water concentration in the liquid phase (ppm)				
	0 h	8 h	24 h	48 h	96 h
0	307.0	-	293.6	-	-
2.5	305.28	328.6	276.3	234.4	169.6
5.0	283.2	197.2	175.7	107.5	108.7
12.5	213.18	60.8	43.9	-	-
25.0	202.8	40.4	34.7	-	-

Effect of the substrate molar ratio (butyric acid/citronellol)

For the chemical synthesis of esters, excess acid is usually involved. This methodology can be also applied in the lipase-catalyzed esterification reaction, as a pattern reaction. However, short chain organic acid may display some inhibitor effects on the enzyme activity[28-30]. To check the effect of butyric acid on enzyme performance, molar ratios between butyric acid (BA) and citronellol (CITRO) in the range of 0.3 - 4.0 were used.

As observed in Fig. 4, no inhibition of the enzyme activity was detected when butyric acid was used in excess. Actually, the esterification progress was even limited by the availability of butyric acid in the reactor vessel. When the molar ratio was 0.3, the synthesis stopped in the first hour, while by increasing the ratio to 0.6 it was allowed to run for another 3 h. Citronellol conversion rates obtained at these molar ratios were 23.8 and 43%, respectively. With a further increase in the molar ratio to 1.2, the reaction attained its completion in 10 h with a 92.9% conversion. For higher molar ratios (2.5 and 4.0), even higher conversions were achieved, up to 97.5% (Fig. 5). However, it seems that high butyric acid concentrations slowed down the reaction rates, and reaction completion was not reached in 10 h. In view of these findings, further experiments were carried out using molar ratios greater than 1.0 and lower than 1.5.

It is also worthwhile to note the shift of the equilibrium towards hydrolysis when the reaction was limited by low levels of butyric acid. Under this condition, there was a build-up of water in the reaction vessel, followed by a slight decrease in the degree of conversion (Table 2).

Effect of substrate polarity

Since the substrate concentration makes a predictable contribution to the overall water content in the reaction mixture, it is expected that a high substrate concentration, and therefore a more hydrophilic substrate, would have a profound influence on esterification performance. To elucidate these findings, reaction mixtures containing 1 mol concentrations of either one or both reactants were used for the ester synthesis with a constant level of Lipozyme (25 mg/mL).

Excess substrate, as such, had little effect, but the degree of conversion was strictly limited by the level of water in the reactor vessel (Fig. 6). This amount tended to increase when the reaction became limited by either the butyric acid or citronellol content. The reverse reaction was most evidenced when the reaction was running at the lowest butyric acid content (molar ratio of BA to CITROL of 0.3:1). Hydrolysis prevailed over synthesis at about 20 h, and continued until all of the citronellyl butyrate was completely hydrolyzed. At the lowest citronellol concen-

Table 2. Monitoring the water content (ppm) in the liquid phase as a function of time for substrates containing different molar ratios of butyric acid and citronellol

Molar ratio BA: CITRO	Water content in liquid phase (ppm)				
	0 h	4 h	8 h	12 h	24 h
0.30	108.7	58.8	82.9	54.7	181.5
0.60	106.0	38.1	78.4	40.0	89.4
1.20	113.2	53.7	37.6	37.7	33.9
2.5	222.8	201.6	103.5	52.8	74.0

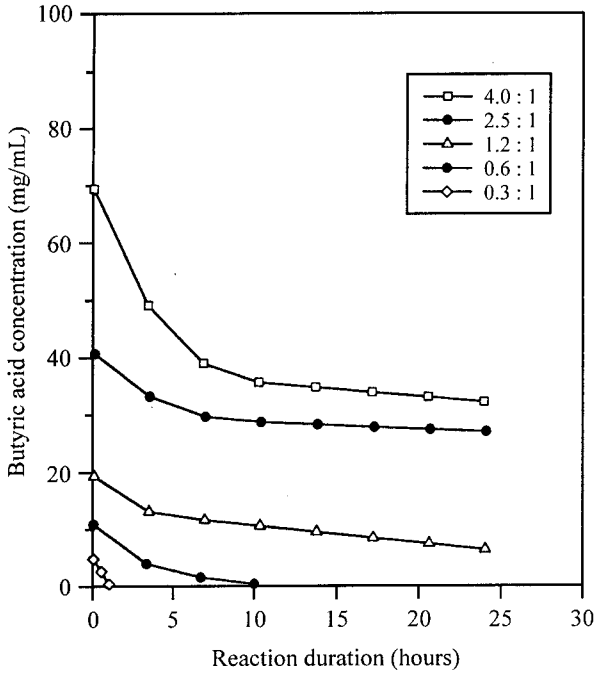

Figure 4. Consumption of butyric acid for different molar ratios of acid to alcohol. Molar ratios of 4:1, 2.5:1, 1.2:1, 0.6:1, and 0.3:1 were used at 40 °C and 25 mg/mL of Lipozyme.

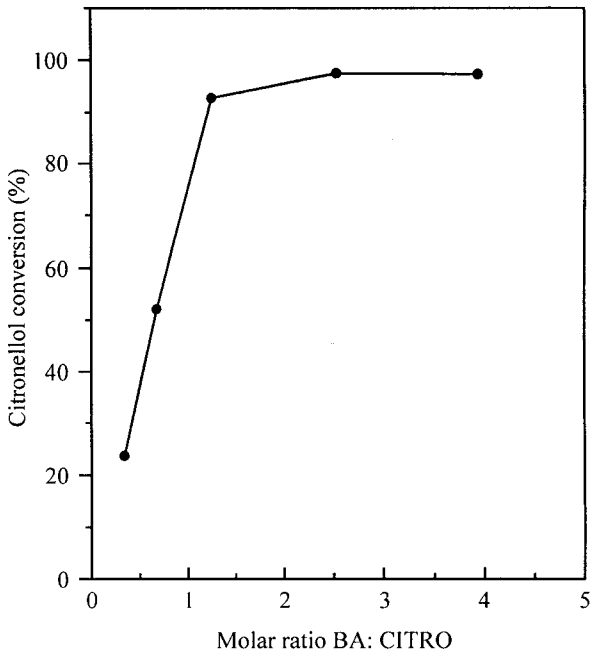

Figure 5. Effects of the ratio of butyric acid to citronellol in the conversion of citronellol after 24 h of incubation with 25 mg/L of Lipozyme at 40 °C.

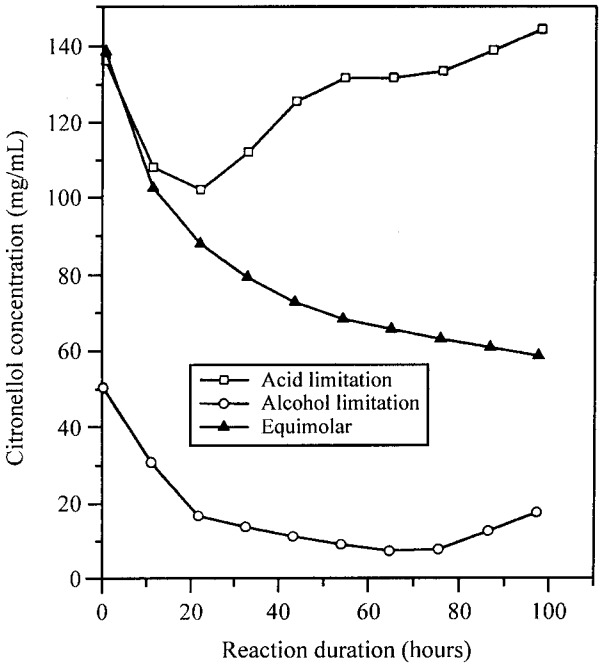

Figure 6. Influence on citronellol consumption on initial concentrations (in mg/mL) at high substrate polarity.

tration (molar ratio of CITRO to BA of 0.3:1), a similar, though less dramatic inhibition trend was observed at about 72 h. In the presence of nearly equimolar amounts of butyric acid and citronellol, ester synthesis continued for a total of 96 h. Two hypotheses could explain this decrease. The first is related to the mechanisms of Lipozyme, which are expected to promote acyl migration of the reaction components[32]. In this way, at the highest substrate concentration, most of the enzyme was acylated with butyric acid, and little free enzyme was available for reaction with citronellol. This also explains the differences in substrate utilization rates, which always have high values for butyric acid. The average value from our data was found to be 1.5 (data not shown). The second hypotheses is also related to the amount of enzyme in the reaction mixture, which was not in a suitable proportion to bind all of the water generated during the reaction. This was confirmed by quantifying the water concentration in the solid phase in two reaction periods (48 and 72 h), with results shown in Table 3.

The water content in the liquid phase is shown in Fig. 7. Batch runs carried out with high substrate concentrations showed different profiles compared to those with low concentrations. First of all, the water content in the liquid phase did not decrease significantly. In addition, for reactions limited either by butyric acid or citronellol, there was a dramatic increase in the water content after 24 h of incubation, with a pronounced effect on the reverse direction of the reaction. At equimolar concentrations, no reverse reaction was observed, although the reaction rate was greatly reduced.

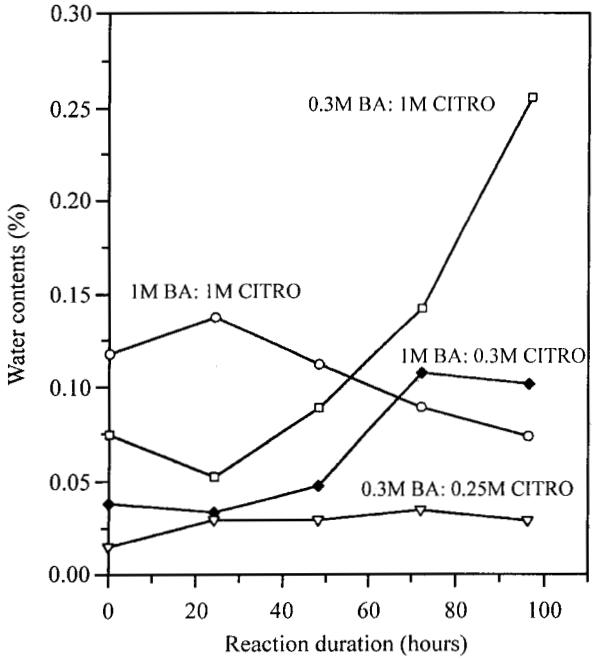

Figure 7. Water concentration in the liquid phase during the course of the reaction at different molar ratios of reactants. Initial concentrations (in mol/L) of butyric acid (BA) and citronellol (CITRO) are shown for each curve.

Table 3. Lipozyme moisture content during esterification of citronellol with butyric acid at different molar ratios.

Initial reactant concentration (mg/mL)		Molar ratio	Lipozyme moisture content (%)	
Butyric acid	Citronellol	BA: CITRO	at 48 h reaction	at 96 h reaction
30.4	150.7	0.3:1	28.3	43.5
109.2	53.5	1:0.3	28.1	46.4
104.6	151.3	1:1	41.0	40.8

These results suggest that the content of Lipozyme is the most powerful factor in the reaction equilibrium position, keeping low water levels throughout the esterification reaction. This also indicates that substrate concentrations make a major contribution to the overall water content in the reaction mixture. Thus, a correlation between the amount of enzyme and the concentration of reactants still needs to be studied to get a better understanding of how the biocatalyst itself can be used as a water buffer for the whole system.

Discussion

The determination of several parameters for the esterification performance using immobilized commercial lipase was carried out. The results demonstrated the feasibility of the production of citronellyl butyrate from citronellol and butyric acid using Novo's Lipozyme as the catalyst. Under the experimental conditions, esterification conversion yields above 95% can be obtained. The molar ratio between butyric acid and citronellol should be greater than 1.0 in order to minimize the competition of citronellyl butyrate as the acyl donor. At this molar ratio, the water formed as a by-product was found to be adsorbed onto the enzyme solid phase, decreasing the water content in the liquid phase. A close relationship between low water content in the liquid phase and conversion of the substrates was obtained. For reactions limited either by butyric acid or citronellol, there was a dramatic increase in the water content at the point where the synthesis stopped. Due to the high hydrophilic property of Lipozyme, its concentration in the reaction media was found to be the most powerful factor in the reaction equilibrium position, by keeping lower water levels throughout the esterification reaction. The main advantage of this feature is to buffer the water activity of the reaction system, so that the required synthesis direction is not inhibited. A combination of Lipozyme and support material could be useful to ensure optimal water content in order to perform the esterification reaction without any further control or removal of the water generated during the reaction. This approach could have a better practical appli-

cation than the utilization of a pair of salt hydrates, particularly when repeated batch use of Lipozyme is envisaged, since the separation procedure of the enzyme and salts will not be necessary.

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