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Production of Citronellyl Acetate in a Fed-Batch System Using Immobilized Lipase

Scientific Note

HEIZIR F. DE CASTRO,* DIOVANA A. S. NAPOLEÃO, AND PEDRO C. OLIVEIRA

Department of Chemical Engineering, Faculdade de Engenharia Química de Lorena, PO Box 116, 12600-000-Lorena, S. Paulo, Brazil

ABSTRACT

Several reports exist in the literature citing the decrease in conversion rates of organic-phase catalytic synthesis reactions when acetic acid is present as a reaction component. This inhibition is thought to result from damage to either the hydration layer–protein interaction or the overall enzyme structure. In this work, the inhibitory effect of acetic acid on lipase enzyme activity was ameliorated by conducting syntheses under acetic acid-limiting conditions in a fed-batch system, resulting in higher product yields. Periodic additions of acetic acid at levels of 40 m*M* or less gave maximum yields of 65% conversion for the reaction of citronellol and acetic acid to form citronellyl acetate. The enzyme used was a fungal lipase from *Mucor miehei*, and was immobilized on macroporous synthetic resin (a Novo lipozyme Novo Nordisk, Denmark). These results represent a fourfold improvement over batch runs reported in the literature for direct esterification of terpene alcohol with acetic acid using lipozyme as a catalytic agent.

Index Entries: Citronellyl acetate; lipase; esterification; fed-batch system.

INTRODUCTION

The use of enzymes in nonaqueous media has become a favored research topic and publications are numerous *(1–3)*. At the research level, difficulties associated with selection of enzymes that are more compatible with nonaqueous media and establishing the somewhat unusual optimal conditions (e.g., enzyme hydration, solvent, and substrate polarity) have

* Author to whom all correspondence and reprint requests should be addressed.

been solved, at least for a few processes (4–6). Among these, bioprocessing using lipases is a well-established, useful method for the preparation of esters either by esterification or interesterification reactions (7–9).

Esters of carboxylic acids are important components of natural aromas, contributing to the flavor in most fruits and many other foods. The lipase-catalyzed synthesis of more than 50 flavoring esters has been described to date (10), and, in principle, the reaction can be carried out in a mixture of alcohol and carboxylic acid with or without solvents, resulting in very high productivities and yields (9–12). An exception is found when acetic acid is used as acyl donor because of its inhibitory effects on lipase esterification activity. Although several commercial lipase preparations have been already screened for their ability to promote synthesis of selected low-molecular-weight esters (water soluble, <C4), the majority of them display very low yields (13–16). Previous results (17,18) show that it is more difficult to prepare acetates in similar conditions than their homologs (butyrates, pentanoates, myristates), for which yields of nearly 100% are attainable (11,12). According to several researchers, the presence of acetic acid in the reaction medium can damage either the hydration layer-protein interaction or the overall enzyme structure, causing reaction inhibition (13–15). Since acetate esters are considered important flavors, several attempts have been made to minimize the inhibitory effect of acetic acid on lipase esterification activity, including the use of alcoholysis reactions (19,20).

A different approach is proposed in this work. By starting the synthesis under acetic acid-limiting conditions followed by periodic additions of acetic acid to the organic medium, higher yields are possible. The system chosen for this study was the esterification of citronellol with acetic acid using fungal lipase from *Mucor miehei* immobilized on macroporous synthetic resin (Novo Nordesk). Lipozyme has been described and characterized throughly (4,6,21,22).

MATERIAL AND METHODS

Materials

The enzyme, Lipozyme IM[20], had an activity of 24 BIU/g (1 BIU corresponds to μmol of palmitic acid incorporated into triolein per min, at standard conditions) and was kindly provided by Novo Nordisk. It was used as supplied (10% w/w moisture content). Reactants (R/S- citronellol and acetic acid) were purchased from Sigma (St. Louis, MO). All substrates were dehydrated before use, with 0.32-cm molecular sieves (aluminum sodium silicate, type 13 X, BHD Chemicals).

Citronellyl Acetate Synthesis

The esterification reactions were performed at 30°C in 250-mL round-bottomed flasks with magnetic stirrer (100 rpm). The initial substrates con-

sisted of citronellol at fixed concentration (240 m*M*) and variable amounts of acetic acid (80–240 m*M*), using heptane as the solvent. Substrates were inoculated with 25% (w/w reactants) of lipozyme *(17)*. To control and monitor the water level in the reaction media, syntheses were carried out in the presence of molecular sieves, as previously described *(23)*. Acetic acid (40–120 m*M*) was periodically added to the reaction medium up to an equimolar ratio of 1:1 between the substrate materials.

Analysis

The reactions were monitored by measuring reactant concentrations by gas chromatography using a 6 ft 5% DEGS on Chromosorb WHP, 80/10 mesh column (Hewlett Packard), and heptanol as an internal standard. Water concentrations in the liquid and solid phases were measured by Karl Fischer method using the Karl Fischer Tritrator (Mettler DL 18). The results were evaluated by calculating the citronellol conversion rates, as follows.

$$\text{Conversion rate (\%)} = \left[(C_0 - C)/C_0\right] \times 100 \qquad (1)$$

where: C_0 = initial concentration of the reactant and C = concentration of reactant at a given time.

Estimation of Partition Coefficients

The partition coefficients (lipozyme/external organic solvent) of citronellol and acetic acid were estimated according to the following equation *(11,18)*:

$$\text{Partition coefficient} = \left[(C_0 - C)/C\right] \times \left[(V_0/(V - V_0)\right] \qquad (2)$$

In order to estimate the lipozyme volume ($V - V_0$), a calibration curve volume of lipozyme vs mass of the lipozyme was established (volume of matrix [cm^3] = 0.41 × mass of lipozyme [g] − 0.025).

RESULTS AND DISCUSSION

Although synthesis parameters can be studied as generic factors, some of them will be very process specific. This is especially true when a more hydrophilic organic is used as substrate. In such systems, biocatalyst deactivation may occur, presumably by either the action of a toxic organic substance or by the potential deleterious effects of liquid–liquid interfaces on the structure of the biocatalyst *(3)*. The actual mechanism of the enzyme inhibition caused by some organic media is not well-understood, although it has been suggested that the reduction of the catalytic activity could be

Table 1
Identification of the Experiment Runs for the Production of Citronellyl Acetate

Run Code	*Reactants*	*Initial Conditions*	*Acetic Acid Additions*		
Run 1	Citronellol	240 mM			
	Acetic Acid	240 mM	0	0	0
Run 2	Citronellol	240 mM			
	Acetic Acid	120 mM	0	0	0
Run 3	Citronellol	240 mM			
	Acetic Acid	120 MM	120 mM	0	0
Run 4	Citronellol	240 mM			
	Acetic Acid	120 mM	60 mM	60 mM	0
Run 5	Citronellol	240 mM			
	Acetic Acid	120 mM	40 mM	0	0
Run 6	Citronellol	240 mM			
	Acetic Acid	80 mM	40 mM	40mM	0
Run 7	Citronellol	240 mM			
	Acetic Acid	80 mM	40mM	40mM	40 mM

associated with conformational changes on the enzyme structure *(1,3)*. This phenomenon appears to be related to the protein–solvent interaction and/or to the removal of water and dehydration of enzyme protein.

In the case of acetic acid, which is more soluble in aqueous phase than in organic media, it is expected that most of the acetic acid would be located in the microaqueous environment of the enzyme. Consequently, the local pH decreases, modifying the enzyme active site and making the reaction nearly impossible.

To investigate the acetic acid tolerance of lipozyme, a set of experiments was performed involving a gradual increase of acetic acid concentration in the reaction medium up to a level in which equimolar amounts of the reactants initially present. The experimental conditions and results are given in Table 1 and Fig. 1, respectively.

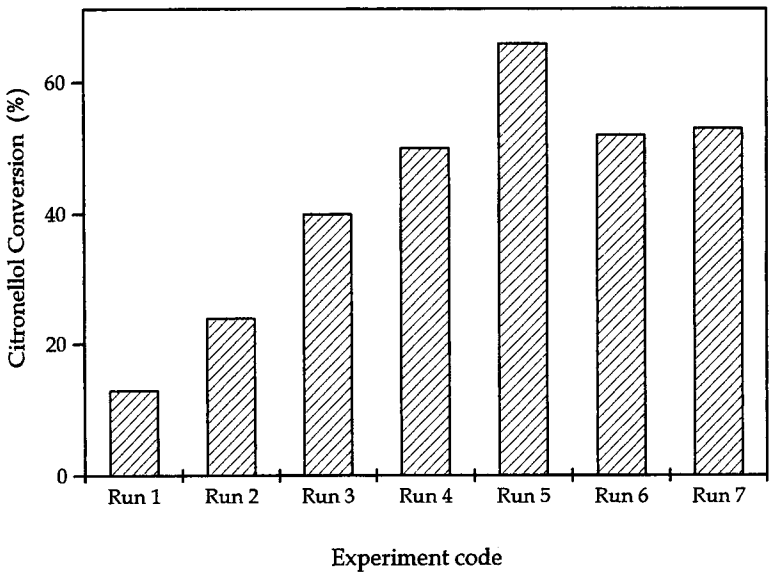

Fig. 1. Final citronellol conversion attained for different proportions of acetic acid added to the reaction medium. Reactions were carried out as described in Table 1. Batch runs 1 and 2, were used as control without any further supplementation of acetic acid.

In the presence of equimolar amounts of acetic acid and citronellol (batch run 1), little ester synthesis occurred. Average citronellol conversions were lower than 15%. Under such conditions, the acetic acid changes the polarity of the reaction medium, which in this turn modifies the partitioning of water between the solid phase (enzyme preparation) and the liquid phase (substrate), resulting in its accumulation on the enzyme solid phase *(17)*. Similar behavior was observed for a different model of study using this enzyme preparation *(24)*. This is also supported by more recent investigations of enzyme activity dependence on substrate concentration in the aqueous layer around the catalyst in which enzymatic reaction occurs *(25)*. According to these authors *(25)*, a correlation between enzyme activity and substrate partition coefficient could be determined. Previous results showed that there is a negative relationship between enzyme activity and substrate-partition coefficient (Ps); that is, the higher the substrate-partition coefficient, the lower the amount of product formed *(18)*. For this particular case, based on the coefficient partitions of each reactant, a substrate partition coefficient value of 11 was estimated, a value favoring the migration of acetic acid to the enzyme preparation (*see* Table 2).

Probably because of this, the esterification performance was improved when acetic acid was used as limiting reactant (batch run 2); however, no further citronellol conversion was attained. Since the esterification is an equimolar reaction, acetic acid should be added up to a molar ratio of 1:1 in order to increase the conversion of citronellol, as performed in runs 3–7.

Table 2
Partition Coefficients Matrix/Heptane of Reactants Estimated According to Eq. 2 at Initial Bulk Concentration of 0.25 *M*, 30°C and 150 rpm.

Reactant	*log P*	*Partition coefficient*
Citronellol	3.9	0.60
Acetic acid	-0.23	6.80
Citronellol + Acetic Acid	1.3	11[a]

[a] Substrate partition coefficient was calculated on the basis of the relation between the partition coefficients of acetic acid and citronellol (*see 18,25*).

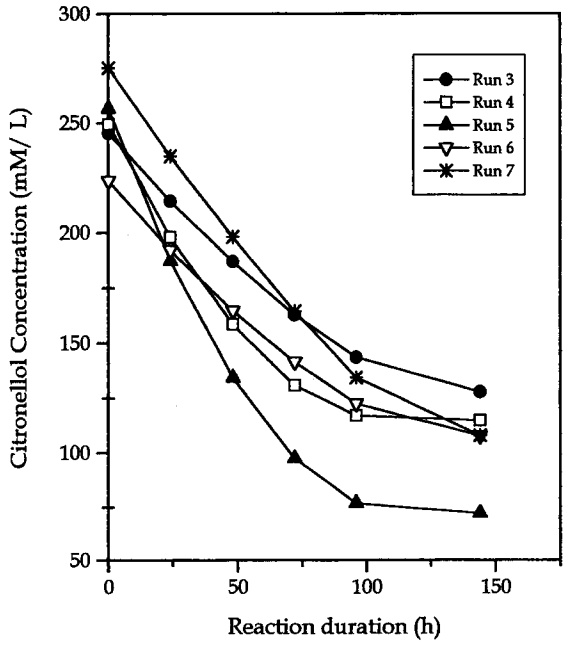

Fig. 2. Progress of the esterification reactions for different acetic acid additions, runs 3–7.

The response for citronellol consumption when different amounts of acetic acid were added at regular intervals (24 h) is shown in Fig. 2. For these runs, there was a significant increase in the citronellol conversion, ranging from 40 to 65%. Better performance was achieved when small levels of acetic acid (1 × 40 m*M* of acetic acid) were added in the reaction medium (run 5).

The concentration profiles of citronellol and acetic acid for run 5 (Fig. 3), indicate that citronellol levels gradually decrease whereas acetic acid sharply decreases in the first 24 h and then reaches a constant value. This

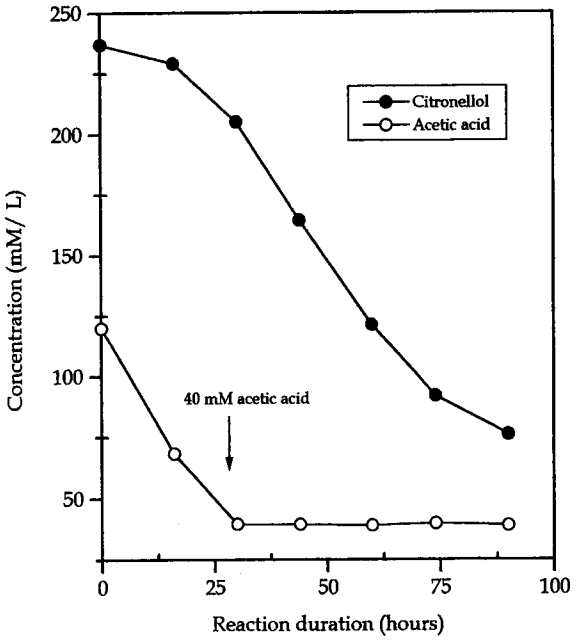

Fig. 3. Concentration profiles of citronellol and acetic acid for run 5 (40 m*M* of acetic acid was added after 24 h incubation).

suggests that during esterification, the consumption of citronellol is greater than the consumption of acetic acid. However, care should be taken on the interpretation of these data as the actual acetic-acid concentration should be greater than the measured one, because the largest amount of acetic acid can be adsorbed by the reaction solid phase, particularly for this enzyme preparation, which has high water-binding capacity *(12)*.

As already mentioned, acetic acid is a potential inhibitor of the reaction. However, the results suggest that there is a concentration range in which satisfactory enzyme activity could be maintained. The approach investigated in this work provides a means to decrease the inhibitory effect of acetic acid on the enzyme activity and enhance the esterication yields to 65%, which is approx 4 times higher than the one obtained in traditional batch runs.

CONCLUSIONS

The presence of acetic acid in the reaction medium can damage either the hydration layer–protein interaction or the overall enzyme structure, causing reaction inhibition. This negative effect can be related to the high polarity of the acyl group, which promotes its migration to the solid-reaction phase (enzyme preparation). This study investigated the feasibility of carrying out the esterification of citronellyl acetate under fed-batch pro-

cess. For the conditions used here, the reaction was found to work satisfactorily under acetic acid-limiting conditions, supplemented with periodic additions at levels of 40 m*M* or less. Such conditions gave maximum esterification yields of 65%. This represents a fourfold improvement over batch runs reported in the literature for direct esterification of terpene alcohol with acetic acid using lipozyme as a catalytic agent. However, taking into consideration the cost of the starting materials, it is still necessary to increase the conversion of citronellol in order to make the application of such a process feasible on an industrial scale.

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