Continuous Lipase-Catalyzed Production of Wax Ester Using Silicone Tubing

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ABSTRACT: Enzymatic synthesis of cetyl palmitate was performed in a solvent-free system at 65°C using immobilized *Candida antarctica* lipase. Batch reactions at controlled water activity showed that the yield could be increased from 88.8 to 99.1% by decreasing the water activity from 1 to 0.05. A continuous reactor configuration was constructed, where two tubular reactors were run in sequence with a separation container in between, in which the water phase was separated from the wax ester phase. The reactor was run for 1 wk at low flow rate (0.005 g/min) with very good operational stability and a productivity of 7.2 g d^{-1} using 0.4 g of biocatalyst. The activity of the individual preparations decreased during operation. The first reactor had only 30% activity left after 1 wk of operation whereas the second reactor showed only a 10% decrease. This difference in enzyme stability is a direct result of the different water activity in the two reactors.

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KEY WORDS: Nonaqueous media, operational stability, packed-bed reactor, water activity.

Lipase-catalyzed esterification is one of the most studied reactions in biocatalysis (1–3). Many enzymatic methods for the production of esters have been described over the last decade (4–7). Moderate reaction conditions, specificity, and high product purity make the biocatalytic routes attractive compared to classical chemical processes. However, the relatively low stability of enzyme must be overcome before lipases become widely used as catalysts in industrial applications. The water formed during the esterification reaction will change the equilibrium position, influence the reaction rate, and reduce enzyme stability. To obtain high thermodynamic conversion and high reaction rate while preventing enzyme deactivation, nearly dry conditions are normally required throughout the reaction. It is important therefore that an enzymatic process include removal of water from the reaction mixture. Toward this goal, different methods of water removal have been proposed. Zacharis *et al.* (8) controlled water content by using salt hydrate pairs. Trani *et al.* (9) developed an open batch reactor with water evaporation at atmospheric pressure while Eigtved *et al.* (10) used a slight vacuum (0.05 atm) to remove the water generated. Recently, Colombié *et al.* (11) tried to use a hydrophilic solvent in the reaction mixture to solubilize the water formed. Mensah *et al.* (12) used a cation exchange resin as a selective water adsorbent. In the present study, we propose a new application of our previously developed water control procedure using silicone tubing (13–15) for the continuous lipase-catalyzed production of wax esters in a solvent-free system.

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MATERIALS AND METHODS

Enzyme preparation. Immobilized lipase SP 435 (Novozyme) from *Candida antarctica* was generously donated by Novo Nordisk A/S (Bagsværd, Denmark).

Tubing. The silicone tubing, 5.0 mm outer and 3.0 mm inner diameter, was from Leewood Marketing AB (Stockholm, Sweden). The polyvinylchloride (PVC) tubing, 4.0 mm outer and 3.0 mm inner diameter, was obtained from Alitea AB (Stockholm, Sweden).

Chemicals. Cetyl alcohol (1-hexadecanol) (96%) and palmitic acid (hexadecanoic acid) (98%) were purchased from Acros (Geel, Belgium), and lauryl alcohol (1-dodecanol) (99%) was from ICN Biochemicals Inc. (Aurora, OH). Capric acid (*n*-decanoic acid; 99%) and the molecular sieves (3 Å) were from Sigma Chemical Co. (St. Louis, MO). *n*-Hexane and the salts LiBr, LiCl, $MgCl_2$, NaBr, and NaCl (analytical grade) were obtained from Merck (Darmstadt, Germany). Other chemicals were of analytical grade.

Gas chromatography. Ester production was measured by gas chromatography (model GC-14A; Shimadzu Corp., Kyoto, Japan) equipped with a flame-ionization detector and a column (2.6 mm, i.d., length 2.1 m) packed with GP10% SP-216-PS on Supelcoport (Supelco, Bellefonte, PA). Helium was used as carrier gas (35 mL/min). The temperature of the injector and the detector was 250°C. For lauryl decanoate, the initial temperature of the column was 170°C for 2 min, and this was then 20°C/min to 190°C. For cetyl palmitate the conditions were: initial temperature 210°C, and then 10°C/min to 225°C. Samples (50 μL) were diluted with 750 μL hexane and analyzed. Standard curves for substrates and products were made to calculate response factors. The percentage conversions were calculated from the amounts of the ester and alcohol peaks.

Reactions under controlled water activity. These experiments were performed inside an incubator thermostated at

65°C. Open reactors containing 20 mg enzyme preparation and 2 g of substrate (equimolar amounts of palmitic acid and 1-hexadecanol) were placed in different sealed containers containing saturated salt solutions LiBr, LiCl, $MgCl_2$, NaBr, and NaCl with water activities (a_w) of 0.053, 0.109, 0.285, 0.495, and 0.747, respectively. For a_w=0 and a_w =1 molecular sieves and pure water were used instead of salt solutions. The ester reaction was run for several days and the conversion measured until equilibrium was reached.

Open-batch reactions. This experiment was performed inside an incubator thermostated at 65°C. Enzyme preparation (100 mg) was placed inside a 10-mL open reactor equipped with a magnetic stirrer (500 rpm) and 4 g of substrate containing equimolar amounts of palmitic acid and 1-hexadecanol was added.

Continuous packed-bed reactions. The apparatus (Fig. 1) was placed in an incubator thermostated at 65°C. The enzyme was packed into silicone or PVC tubing. Substrate containing equimolar amounts of palmitic acid and 1-hexadecanol were pumped through the column using a peristaltic pump (model p-3; Pharmacia Fine Chemicals, Stockholm, Sweden).

Stability measurements. Samples (about 20 mg of enzyme preparation) from the inlet side were removed, washed three times at room temperature with 3 mL toluene for 20 min to dissolve residual cetyl palmitate and the substrates. The wash solution was filtered, and 5 mg of the dried preparation was added to 4 mL hexane solution containing 100 mM capric acid and 100 mM lauryl alcohol. The reactions were performed at 25°C in sealed containers on a shaker, 180 rpm. Samples (50 µl) were withdrawn, and the initial esterification rate was determined. The relative activities were calculated by dividing the initial reaction rates of the enzyme sample from the column by the initial reaction rate of unused enzyme.

Water titration. The water concentrations in the reaction medium were determined on a 684 Karl Fisher coulometer from Metrohm (Herisau, Switzerland). The sample volumes were 50 µL.

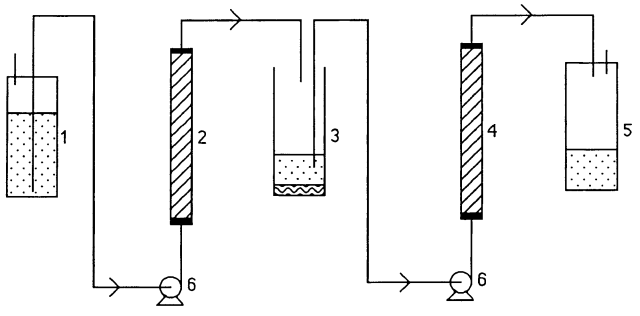

FIG. 1. Experimental set-up for the continuous lipase-catalyzed production of wax ester. 1, substrate container (equimolar amount of palmitic acid and cetyl alcohol); 2, polyvinylchloride (PVC) tubular reactor packed with 200 mg enzyme preparation; 3, intermediate container, upper phase: 88.8% ester, lower phase: water; 4, silicone tubular reactor packed with 200 mg enzyme preparation; 5, product container: 99.1% ester; 6, peristaltic pump. The flow rate was 0.005 g/min.

RESULTS AND DISCUSSION

Wax ester production. Wax ester of cetyl alcohol and palmitic acid was synthesized using a commercial immobilized lipase preparation. Reactions were run in a solvent-free system simply by mixing the two substrates and raising the temperature to 65°C in an incubator. There was a small spontaneous synthesis of ester if the substrates were left a long time in the incubator (less than 1% per day). This was avoided by regularly making fresh substrate solution.

Batch reaction. When the reaction was run in an open vessel inside the incubator, it showed a clear two-stage action (Fig. 2). The reaction was quite fast and reached a steady-state at 88.8% conversion within 30 min. The initial reaction rate was approximately 19.2 µmol/min/mg enzyme preparation. After further incubation, the yield started to increase from the steady-state level, and after 7 h, the yield was 99.1%. This phenomenon is due to the fast enzymatic reaction in comparison to the relatively slow removal rate of water and thus, an accumulation of water occurs. The reaction mixture becomes water-saturated quickly and at 88.8% conversion, 25 mg water/mL of reaction mixture is produced and a separate water phase forms, giving a water activity of 1 in the system. After prolonged incubation, the extra water is slowly removed by evaporation and after 80 min, the water content decreases and wax ester production proceeds. The removal of water and the enzymatic reaction continues until most of the water is removed and the water activity becomes low. At the end of the reaction the water content was approximately 0.3 mg/mL.

A separate experiment was performed with a reaction mixture taken from the steady-state level (88.8% conversion and

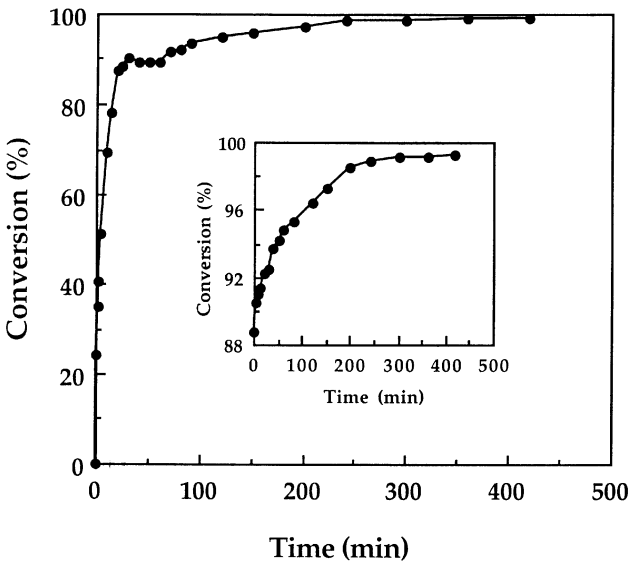

FIG. 2. Esterification reaction in open batch reactor. The reactor contained 100 mg *Candida antarctica* and 4 g (8 mmol) of substrate. Inset: The reactor contained 100 mg *C. antarctica* and 4 g of water-saturated reaction mixture containing 88.8% ester.

water saturated) which contained 2.6 mg/mL dissolved water. In this case, the reaction started as a water-saturated reaction mixture but there was no bulk water phase. To this solution the same amount of enzyme as used previously was added, and the course of the reaction followed. Water removal immediately reduced the water activity and the enzymatic reaction started (insert in Fig. 1). The progress curve for this reaction can be superimposed on the part of the large graph starting from 80 min. The "initial" reaction rates in these cases are 0.12 and 0.13 μmol/min/mg enzyme preparation, respectively. These rates are 100-fold lower than in the beginning of a reaction (Fig. 1). This is due to the lower substrate concentration (10-fold), high product concentration (1.4 M), high water activity (approximately 1), and possibly an increase in viscosity.

a_w controlled reactions. Batch reactions were run under controlled a_w to understand the effect of water content on the reaction yield at thermodynamic equilibrium. Reducing a_w from 1 to 0.053 lowers the water content to 0.3 mg/mL and increases the yield to 99.1% (Table 1). When molecular sieves were added to the reaction mixture the water content decreased and the yield was 100%. The equilibrium constant (K_0), expressed in

$$K_0 = a_w \cdot [\text{ester}]/[\text{acid}]\cdot[\text{alcohol}] \quad [1]$$

units of M^{-1}, can be calculated using the concentrations of substrates/product and the water activity according to Valivety *et al.* (16). This constant sharply increases as the a_w of the system decreases (Table 1). This has been demonstrated before and is an effect of the polarity of the product mixture (8,17). In the present case this phenomenon is even more pronounced since the K_0 increases ninefold from water activity 1 to 0.053. The wax ester mixture containing only 1% of substrates is much more hydrophobic than the wax ester containing 10% of substrates, and this fact will increase the equilibrium constant and promote the synthesis. From the results it can be calculated that running open vessel reactions in the incubator will give an equilibrium mixture that corresponds to an atmosphere of a_w of around 0.05.

Continuous reaction. Instead of a batch reaction a continuous wax ester synthesis was desired. A reactor configuration of two tubular reactors, containing the immobilized enzyme, was constructed, similar to that described earlier (15). In our case the first tubular reactor was made out of PVC tubing, which is not permeable to water (Fig. 1). The effluent from this reactor was collected in a container. Because the amount of water produced exceeded the water solubility, a separate water phase was formed in the first tubing reactor. Accordingly, water droplets eluted together with the ester product mixture and a separate water phase formed in the container. The organic phase containing wax ester and substrates and dissolved water was pumped into the second tubular reactor, made of silicone tubing. In this reactor the reaction continued because water is permeable through the walls of the tubing. The effluent of this reactor was in equilibrium with the atmosphere in the incubator.

First, the two reactors were studied separately. The flow rate of substrates into the PVC reactor was varied. The yield of ester decreased slowly as the flow rate increased from 0.02 to 0.06 g/min (Fig. 3). The second reactor (fed with the product mixture from reactor 1) showed a similar behavior (Fig. 3). The flow rate of the second reactor was reduced since a smaller amount of enzyme was used. The yield was fairly constant at flow rates less than 0.01 g/min. Increasing flow rates up to 0.06 g/min decreased the yield from 99.1 to 95%. The regions at low flow rates demonstrate that the reactors under these conditions are under thermodynamic control, yielding 88.8 and 99.1% conversions, respectively.

Two-stage reactor. The two tubular reactors were run in series in an experiment over 1 wk. The flow rate was chosen to be low, 0.005 g/min, in order to obtain high conversion of ester (approximately 7 g of product was produced per day). Over this period, the ester yields in the first and second reactor were constant corresponding to equilibrium (Fig. 4). This shows that during the 7 d of reaction, the operational stability of the reactors was very good.

TABLE 1
Equilibrium Values at Different Water Activity

Water activity a_w	Water concentration (mg/mL)	Ester yield (%)	K_0[a] (M^{-1})
0.05	0.34	99.1	397
0.11	0.42	98.5	310
0.29	0.84	96.0	111
0.50	1.2	93.9	81
0.75	2.2	90.6	50
1.0	2.7	88.8	46

[a]K_0, equilibrium constant.

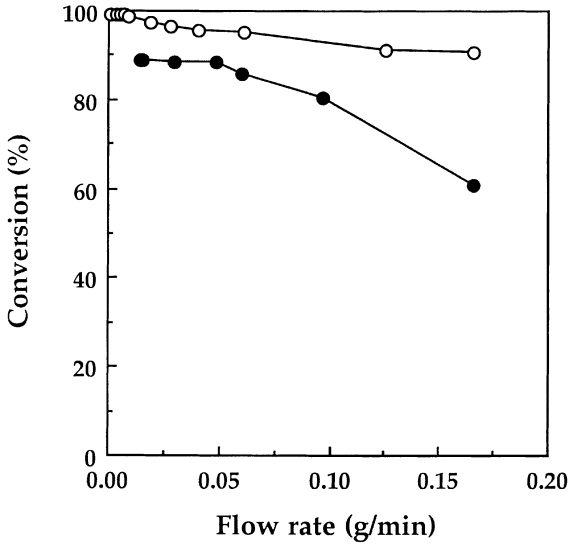

FIG. 3. Esterification at different flow rates, in tubular reactor containing 400 mg *C. antarctica* packed in PVC tubing (●), and 100 mg *C. antarctica* packed in silicone tubing (○). In this reaction the water-saturated substrate consists of 88.8% ester. For abbreviations see Figures 1 and 2.

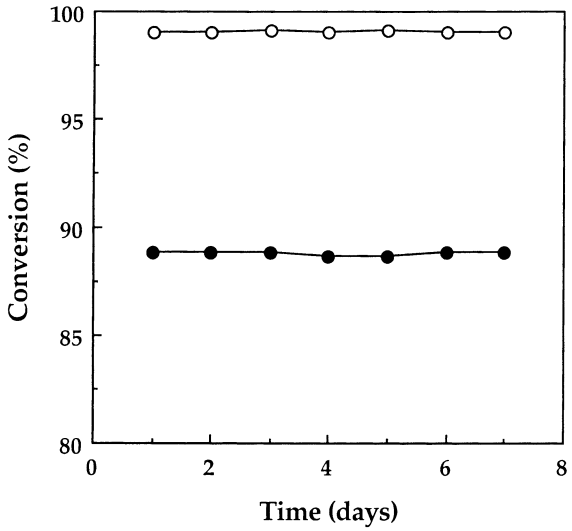

FIG. 4. Operational stability in PVC tubular reactor (●) containing 200 mg *C. antarctica* and in silicone tubular reactor (○) containing 200 mg *C. antarctica* when used as described in Figure 1. The flow rate was 0.005 g/min. For abbreviations see Figures 1 and 2.

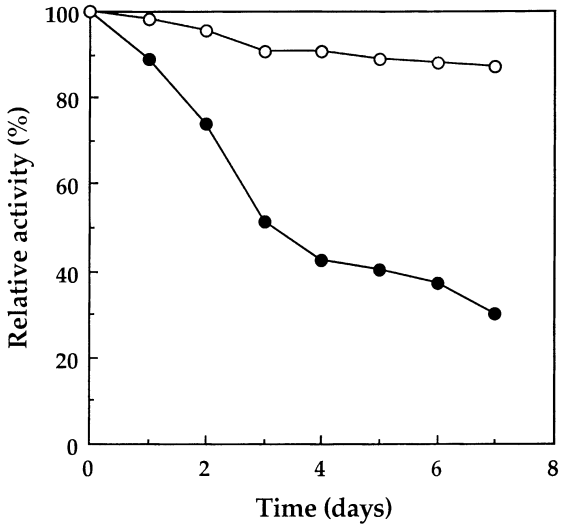

FIG. 5. Relative enzyme stability in PVC tubular reactor (●) and silicone tubing (○) when used as described in Figure 1. The flow rate was 0.005 g/min. See Figure 1 for abbreviation.

Stability of the two reactors. In a separate experiment, samples of the enzyme preparation from the two reactors were withdrawn. The samples were washed and the residual catalytic activity determined using standard reaction conditions. The two reactors showed a different behavior. The specific activity of the preparation decreased for the PVC reactor (Fig. 5). After 7 d, the enzyme activity was 30% compared to 87% for the silicone preparation. Obviously, the stability of the enzyme depends on a_w. The first reactor is run under water-saturated conditions, whereas the second works under lower a_w. a_w is known to influence the thermal stability of an enzyme preparation (18). The decreased stability of the first reactor also can be an effect of the water droplets formed in the reactor, which might denature the enzyme in the interface formed or even dissolve it on its way through the reactor.

Production and stability aspects. The operational stability of the reactors was very good. No decrease in yield was observed during 1 wk of operation. During this time, 50 g of product was produced using 0.4 g of biocatalyst. Looking at the performance of the enzyme preparation in the two separate reactors, one can conclude that the second reactor showed a satisfactory stability (13% decrease in enzyme). The first reactor loses up to 70% of its enzymatic activity over the same period; however, no decrease in product yield is detected even though two-thirds of the enzyme is deactivated. The reactors are run at low flow rates, and there is simply too much biocatalyst still active. Samples for activity determination were collected from the inlet side of the reactors. In the two reactors there are different a_w gradients along the length of the reactors. The first reactor goes from low to high a_w while the second has reversed a_w conditions. Therefore, one can say that the decrease in enzyme activity in the first reactor is underestimated (samples taken at the lowest a_w) whereas the second reactor is overestimated (samples from the highest a_w). Despite these facts, the data give a good indication of the potential problem of enzyme deactivation especially in the first reactor.

The esterification could be performed in a single silicone tubular reactor. In this instance, the yield was similar to the yield of the two-stage reactor. However, with two-stage reactor configuration it is possible to replace the biocatalyst in the first reactor without changing the biocatalyst in the second reactor. This results in less enzyme usage overall.

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