The Influence of Water on the Synthesis of n-Butyl Oleate by Immobilized *Mucor miehei* Lipase

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The influence of initial water concentration on the synthesis of n-butyl oleate was investigated. The synthesis was done with immobilized *Mucor miehei* lipase—Lypozyme™—at various reaction conditions. The activity of the enzyme is lower at higher amounts of water. Initial reaction rates, as well as equilibrium conversion, increase at low initial water concentration. Optimal water concentration for the activity of immobilized lipase is temperature dependent at the pressure of 1 bar. Low initial water concentration barely effects equilibrium esterification at 0.032 bar. At high initial water concentrations equilibrium conversion, as well as initial reaction rates, decrease at both pressures.

KEY WORDS: Batch stirred tank reactor, biotechnology, esters synthesis, immobilized lipase, lipase, *Mucor miehei*, n-butyl oleate, water influence.

Esterification between oleic acid and n-butanol can be performed in a biocatalytic way (1,2) since it is the reverse reaction of hydrolysis. Most synthesis of long chain fatty acid esters and glycerides were done with the free lipase (3,4) in a two phase system which is necessary for lipase action. Immobilized lipase acts on a carrier surface which replaces the lipid-water interface. Water may be present in very small amounts in such a system, and the lipase is still active. There have been some reports (3,5) on the influence of water on the free lipase activity in the synthesis of long chain fatty acid esters and glycerides. On the contrary (5,6), it was found that the free porcine pancreatic lipase acts in nearly anhydrous organic solvents and catalyzes the reactions of esterification and transesterification.

From our previous reports (1,2,7) it is obvious that the lipase from *Mucor miehei* immobilized on a macroporus anion exchange resin catalyzes the esterification of n-butyl oleate in an almost nonaqueous media. No organic solvent was necessary for the synthesis. The immobilized lipase may be reused, depending on the half-life of the enzyme (7). After a certain amount of time, purification of the immobilized enzyme is necessary before its reuse. Drying the beads with immobilized enzyme is a part of this procedure. It is very important to know how much water must be added to the enzyme preparation before its application.

Initial water concentrations significantly affected the equilibrium of the hydrolysis in reversed micelles. The maximum fractional conversion of ester bond to fatty acid and alcohol moiety generally increased as the initial water concentration increased (8). Because esterification is the reverse, it is expected that the equilibrium conversion to ester will decrease as the initial water concentration increases.

It has been reported that the amount of water present in the reaction mixture has very little effect upon the synthesis of n-butyl oleate when the reaction is catalyzed with the purified lipase from *Pseudomonas fragi* 22.39 B (3). The influence of water present on the initial reaction rate was not investigated.

In the present paper the effect of initial water concentration on initial reaction rates and equilibrium conversion will be discussed. Esterification was carried out at two different pressures—1 bar and 0.032 bar. N-butanol as substrate inhibits enzymes at high concentrations. At low pressure it evaporates from the reaction mixture. Therefore, esterification at 0.032 bar was investigated for the comparison with esterification at 1 bar, where almost all of the n-butanol is present in the reaction mixture.

EXPERIMENTAL PROCEDURES

Enzyme preparation. For the synthesis of n-butyl oleate, *Mucor miehei* lipase was used as a catalyst. The lipase—Lipozyme™—immobilized on a macroporus anion exchange resin, was kindly donated from Novo Industri A/S (Copenhagen, Denmark). The enzyme beads contained about 10 w/w% of water.

Lipase activity. Lipase activity was measured by Novo Industri A/S. The activity of the Lipozyme preparation used for our synthesis was 23 BIU/g. One Batch Interesterification Unit (BIU) corresponds to 1 mole of palmitic acid incorporated in triolein per min at standard conditions (9).

Chemicals. Oleic acid was purchased from Merck (Darmstadt, Germany). All other chemicals, including n-butanol as substrate, were from Kemika Zagreb (Yugoslavia).

Synthesis of n-butyl oleate in a batch stirred tank reactor (BSTR). The reaction mixture contained 47 mmol of oleic acid, 47 mmol of n-butanol, 1.04 g of enzyme preparation and various amounts of water. The mixture was stirred in a 250-mL, round bottom flask with a magnetic stirrer, and heated up to the desired temperature in a water bath. At certain time periods samples were taken from the reaction mixture and the level of free fatty acid (FFA) was determined. From these values, concentrations of n-butyl oleate were calculated. After 3 hr the reaction was stopped by separation of the enzyme beads from the reaction mixture with filtration.

Determination of the FFA content. First, 0.1 g of sample of reaction mixture was diluted in 20 mL of 0.1 wt% phenolphthalein solution in ethanol and titrated with NaOH. Then, the amount of FFA content was calculated from the equation:

$$\%\mathrm{FFA} = (V \times N \times \mathrm{Mol.a.})/(10 \times w)$$
$$= (V \times 0.1 \times 282.47)/(10 \times w)$$

$$\%\mathrm{FFA} = 2.8247 \times (V/w)$$

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V, volume of NaOH, c(NaOH)= 0.1 mol/L; and w, weight of sample of the reaction mixture (g).

RESULTS

Synthesis of n-butyl oleate with immobilized *Mucor miehei* lipase in the BSTR was done with various initial water concentrations. The reactions were carried out at different temperature-pressure combinations: 70°C/1 bar, 50°C/1 bar, 50°C/0.032 bar, 20°C/1 bar and 20°C/0.032 bar.

Figures 1a and 1b show the variation of ester concentration vs time at a pressure of 1 bar and temperature of 50°C at various initial water concentrations. From this Figure it is seen that lipase activity depends on the amount of water which is present in the reaction mixture. The same effect also was found with all other reactions that were examined.

From the slopes of the plots of ester concentration vs time, initial reaction rates for all reaction conditions were calculated. The first nine min of the reaction were neglected because of the heating and mixing problems at the beginning of the reaction (substrates and water were not equally distributed in the reactor). Initial reaction rates at 1 bar increased slightly with an increase in initial water concentration until the maximum of the curve. With further increases in the initial water concentration, the initial reaction rates decrease (Fig. 2a).

At 20°C, very low initial water concentrations influence the initial reaction rate, which increases slightly till the constant value, and then decreases quickly. At 50°C and 70°C initial reaction rates increase until relative high initial water concentrations. Their increase is almost the same at both temperatures.

At the pressure of 0.032 bar, initial water concen-

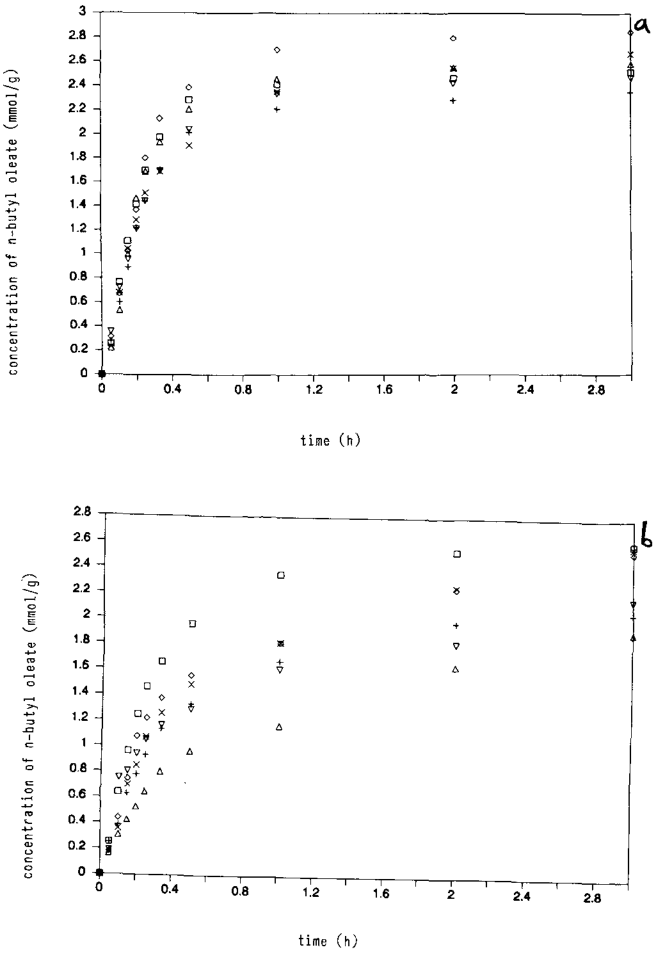

FIG. 1. Concentration of n-butyl oleate vs time for various initial water concentrations. The reaction mixture consisting of oleic acid, n-butanol, immobilized lipase and various amounts of water was stirred with a magnetic stirrer and thermostatted for 3 hr (details are described in the text) at 50°C and 1 bar. Initial water concentrations in mol % were: a, □, 4 + 9; ◇, 12.5; Δ, 15.5; ∇, 19; ×, 23; and b, □, 26; +, 37; ◇, 44.5; ×, 52; and ∇, 58.5.

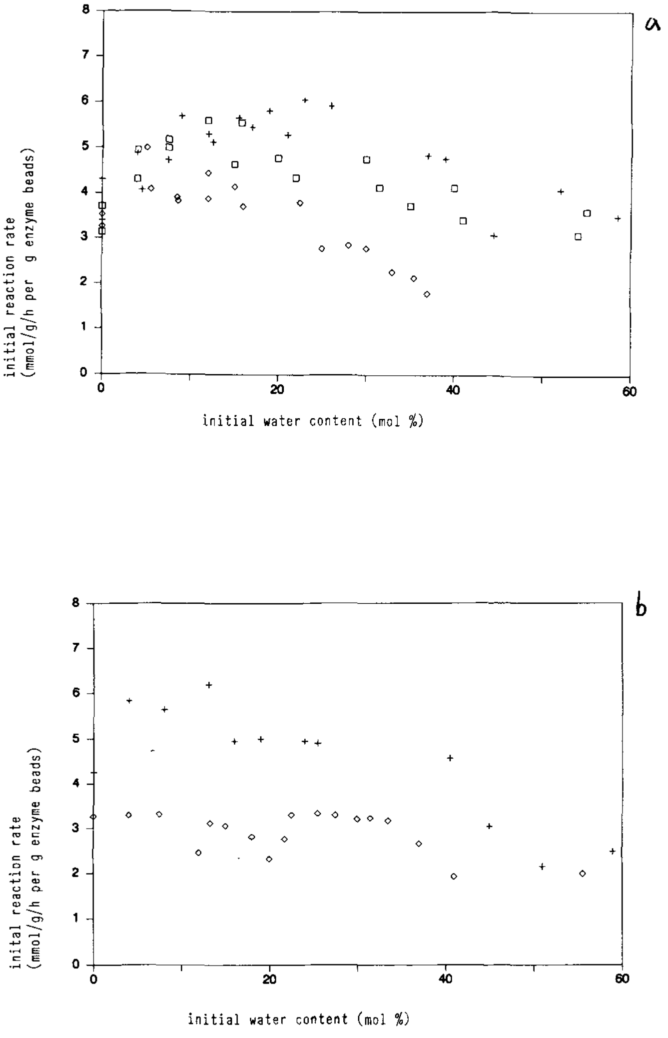

FIG. 2. Initial water concentration influence on the initial reaction rates at 1 bar (a) at 20°C (◇), 50°C (+), 70°C (□); and at 0.032 bar (b) at 20°C (◇) and 50°C (+). (Calculated points from the experimental data are missing for the sake of clarity). Initial reaction rates were calculated from the slopes of plots of concentration of n-butyl oleate vs time for various initial water concentrations (Figs. 1a, b, e, and others that are not included in the article).

tration barely have any influence on initial reaction rates at both investigated temperatures (Fig. 2b). The reason is low pressure at which water was evaporated from the reaction mixture. The highest initial reaction rates are at 50°C, regardless of the initial water concentration in the reaction mixture.

Equilibrium esterification also increases with increasing initial water concentration until the maximum of the curve, and then it decreases to a minimal constant value (Fig. 3). At 70°C, the influence of low initial water concentration is higher than at 20°C and 50°C. This is probably due to the n-butanol evaporation that occurs at high temperatures if no water is added to the reaction mixture. Concentration of n-butanol rises in the reaction mixture with the addition of water because of the azeotropic mixture of water and n-butanol. At low temperatures n-butanol concentration in the reaction mixture is almost the same with or without addition of water. Therefore, the influence of low initial water concentration is smaller. Optimum amounts of water at the pressure of 1 bar are: 70°C, 10 mol %; and 20°C and 50°C, 15 mol% and 20 mol %, respectively. These values do not include the amount of water which is incorporated in enzyme beads. Commercial available enzyme preparation Lipozyme (from Novo Industri A/S) includes approximately 10 w/w% of water.

At the pressure of 0.032 bar, almost no influence of low initial water concentration on equilibrium conversion is observed. Equilibrium concentration of n-butyl oleate is almost constant, regardless of initial water concentration at low values; and at relatively high initial water concentration it starts to decrease.

DISCUSSION

The synthesis of the long chain fatty acid ester is the reverse reaction of hydrolysis. There are two end products—fatty acid ester and water. Therefore, the addition of water at the beginning of the reaction affects the equilibria. For the enzymatic synthesis of n-butyl oleate the equilibrium conversion increases as the initial water concentration increases. With further increases of the initial water concentration, the equilibrium conversion decreases. Two effects are fused in this case—water affects the equilibria of the reaction, and the immobilized *Mucor miehei* lipase activity decreases with too much water present in the reaction mixture.

Bell *et al.* (10) reported that the absence of water increases esterification, while Zaks and Klibanov (11) showed that the water content does not influence the activity on *Mucor sp.* lipase. Nishio *et al.* (3) mentioned that the synthesis of n-butyl oleate was not affected very much by the amount of water in the mixture.

Our investigations confirmed that the amount of water present in the reaction mixture at the beginning of the reaction is very important for the synthesis of n-butyl oleate, which is carried out at 1 bar. When using dry immobilized *Mucor miehei* lipase for this esterification, the optimum amount of added water is very dependent on the temperature at which esterification is carried out. On the contrary, at low pressures, initial water concentration is not important for esterification.

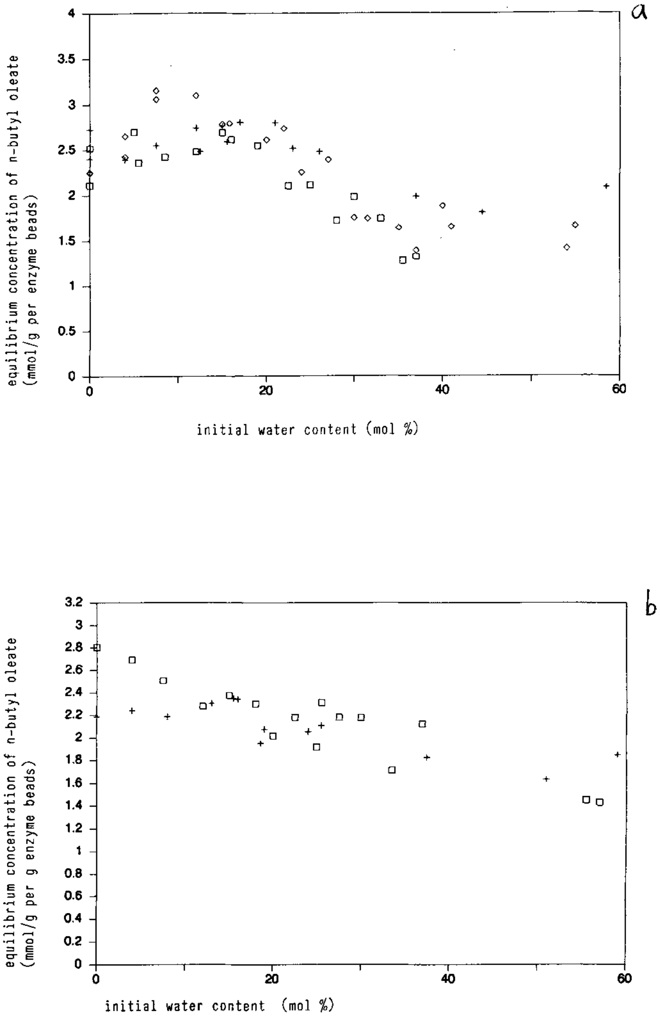

FIG. 3. The dependence of the equilibrium production on n-butyl oleate on initial water concentration. The reactions were carried out for 3 hr at the pressure of 1 bar (a) at 20°C (□), 50°C (+), 70°C(◇); and at 0.032 bar (b) at 20°C (□) and 50°C (+).

Because of the lower enzyme activity at higher amounts of water, initial reaction rates decrease with an increase of water. This relation is also dependent on the temperature at which the reaction takes place. Previously we reported that maximum initial reaction rates are at 50°C without the addition of water (7). This observation was confirmed with this work. With reaction at 50°C, the reaction rate is maximal with respect to other temperatures, regardless of the amount of water that is present in the reaction mixture.

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