Parameter Optimization for the Enzymatic Hydrolysis of Sunflower Oil in High-Pressure Reactors

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ABSTRACT: This study is concerned with the hydrolysis of sunflower oil in the presence of lipase preparation Lipolase 100T (*Aspergillus niger* lipase). Supercritical carbon dioxide was used as a solvent for this reaction. In a high-pressure stirred tank reactor operated in a batch mode, the effects of various process parameters (temperature, pressure, enzyme/substrate ratio, pH, and oil/buffer ratio) were investigated to determine the optimal reaction rate and conversion for the hydrolysis process. The optimal concentration of lipase was 0.0714 g/mL of CO_2-free reaction mixture, and the highest conversions of oleic acid (0.193 g/g of oil phase) and linoleic acid (0.586 g/g of oil phase) were obtained at 50°C, 200 bar, pH = 7, and an oil/buffer ratio of 1:1 (w/w).

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KEY WORDS: High-pressure, hydrolysis, lipase, sunflower oil, supercritical carbon dioxide.

The production of FA from TG is very important industrially (1). The products find applications in soap and detergents, cosmetics, pharmaceuticals, and foods. Typically, TG are hydrolyzed by steam splitting (Colgate–Emery method), which requires high temperature and pressure as well as subsequent high-pressure distillation steps for the downstream separation of FA. This process is costly, energy-consuming, environmentally unfriendly, and in some cases hazardous because of the extremes of processing conditions.

Since the beginning of the 20th century, it has been known that lipases (2–4) also can catalyze the hydrolysis of fats (5). Fat splitting, with lipase as a catalyst, is advantageous compared to a conventional process due to low energy consumption, high product quality (1,6), and greater safety. In contrast to the conventional high-pressure hydrolysis of fats where the reaction takes place in a one-phase system, the enzymatic reaction takes place at the phase boundary between fat and water (7). Combining enzymes with supercritical fluids (SCF) makes use of the benefits from both enzymes (higher reaction rates, better product purity, safer process, etc.) and supercritical fluids (higher reaction rates, better transport properties, etc.). Lipase-catalyzed reactions in SCF have been reported by numerous investigators (8–10). Supercritical media retain good enzyme activity and stability (11) and can be good solvents for hydrophobic compounds. An additional benefit of using enzymes in SCF is that it provides a very convenient way to recover products or nonreacted components. The most useful temperature range of supercritical carbon dioxide (SC CO_2) and the typical low operating temperatures of enzymes overlap each other.

The system used in our research was the hydrolysis of sunflower oil in SC CO_2, catalyzed by the lipase preparation Lipolase 100T. The reaction was performed in a high-pressure batch stirred tank reactor (HP BSTR), with the aim of studying the influence of reaction parameters on the yield and initial reaction rates.

EXPERIMENTAL PROCEDURES

Enzyme preparation. Lipolase 100T, a nonimmobilized preparation of *Aspergillus niger* lipase, was kindly donated by Novo Nordisk A/S (Bagsvaerd, Denmark).

Chemicals. Sunflower oil was purchased from Oljarica Oil Factory (Kranj, Slovenia) (linoleic acid content was 64.6% and oleic acid content was 21.1%; determined by GC). Since the predominant FA in this oil were linoleic and oleic acids, their release was the focus of all experiments. Buffers employed were 0.1 M potassium phosphate, pH 6; 0.1 M potassium phosphate, pH 7; and a potassium–sodium phosphate buffer consisting of, per liter, 0.5 mL KH_2PO_4 (9.078 g/L) and 9.5 mL $Na_2HPO_4 \cdot 2H_2O$ (11.88 g/L), pH 8. All other chemicals were from Merck (Darmstadt, Germany).

CO_2 (99.95 vol% pure) was supplied by Messer MG (Ruše, Slovenia).

Determination of lipase thermal stability. The crude enzyme preparation was incubated at different temperatures for 24 h in an autoclave at 300 bar in SC CO_2. After slow depressurization, lipase activity was determined by the method described below.

Determination of residual lipase activity. Lipase activity was measured by the following method: After treatment (described above) with SC CO_2, 0.1716 g of lipase was added to a mixture consisting of 2 g of sunflower oil and 2 g of phosphate buffer. The mixture was first adjusted to and held at 50°C and stirred with a magnetic stirrer. The reaction was carried out at 50°C and started by lipase addition. After 60 min, the reaction mixture was centrifuged to separate the oil and buffer phases and to stop the reaction. The amount of FFA was determined by titration (12), and the amounts of free oleic and free linoleic acid were determined by HPLC (13).

(i) Hydrolysis in a HP BSTR. The design of the batch-operated system is shown elsewhere (10). The volume of the reactor,

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which was designed for operation at 500 bar, was 80 mL. The reaction mixture with desired ratio of sunflower oil/phosphate buffer was stirred by magnetic stirrer and heated to the desired operational temperature in an oil bath. Various enzyme amounts were added. Dry CO_2 was then pumped into the reactor to the desired pressure. The reaction medium was monophasic. During the reaction, samples were taken from the reactor, and the amount of FFA was determined.

Each data point represents the average of at least two measurements, and the average of three measurements in cases where problems with operation at high pressure appeared.

RESULTS AND DISCUSSION

Thermal stability of Lipolase 100T. Before its use in SC CO_2, Lipolase 100T was tested for its activity in this medium. Because temperature plays a great role in enzyme activation/deactivation, the thermal stability of Lipolase 100T in SC CO_2 was studied. Enzyme was incubated in SC CO_2 at defined temperatures (between 20 and 80°C) and 300 bar for 24 h. After slow depressurization ($\Delta P/\Delta t$ = 2.5 bar/min), residual activity of the lipase was measured. Activity increased with temperature from 20 to 50°C and was optimal at 50°C. With a further temperature increase, a rapid activity decrease was observed. These changes are probably the result of thermal activation/deactivation of the enzyme with changes in the water distribution in the system. The crude lipase preparation contained 1.8% (w/w) water (measured by the Karl–Fisher method), whereas the SC CO_2-incubated lipase at 70°C contained only 0.82% (w/w) of water. Enzymes need a certain amount of water for their activity and if it is stripped away, the activity of the biocatalysts falls. It has been found that the rate of conformational changes in proteins during thermal inactivation essentially depends on the water content; in turn, inactivation is followed by changes in protein hydration (14). However, another reason for the decreased residual lipase activity at temperatures above 50°C could also exist. A pH-induced inactivation, due to the increased solubility of CO_2 in the microaqueous phase of the enzyme with temperature, could take place.

Hydrolysis in an HP BSTR. (i) Influence of enzyme/substrate ratio on the equilibrium conversion. Enzymes lower the energy of activation and, in doing so, the rates of enzyme-catalyzed reactions become higher. The level of increase in reaction rates is dependent on the concentration of biocatalyst in the reaction mixture. However, at higher enzyme concentrations the degree of reaction rate increase becomes lower and lower, until saturation with the enzyme is reached. At this enzyme level, a further increase in biocatalyst concentration does not increase the reaction rate.

The hydrolysis of sunflower oil by Lipolase 100T in an HP BSTR was conducted at 50°C, 200 bar, and at various enzyme concentrations to determine the optimal amount of biocatalyst at defined conditions. The reaction mixture was stirred with a rotational speed of 600 rpm. Figure 2 shows the concentration of linoleic acid as a function of time at various enzyme concentrations. From Figure 2, we see that of the enzyme levels examined, the maximal linoleic acid concentration was reached at 0.0714 g enzyme/mL of CO_2-free reaction mixture. With a higher lipase concentration in the reaction mixture, the concentration of FFA was not increased. The results for release of oleic acid were identical to those for linoleic acid (data not shown). On the basis of these results, an enzyme concentration of 0.0714 g/mL of CO_2-free reaction mixture was chosen for further experiments. An equilibrium conversion of 92% was achieved after 48 h.

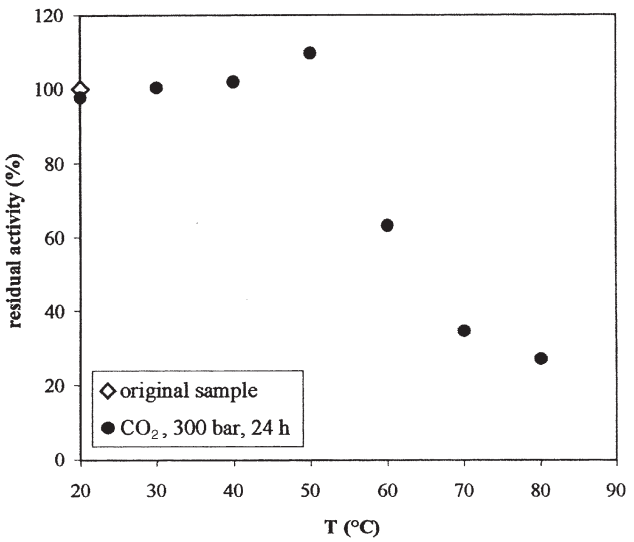

FIG. 1. Residual activity of Lipolase 100T (*Aspergillus niger* lipase) after 24 h incubation at different temperatures in supercritical carbon dioxide (SC CO_2) at 300 bar.

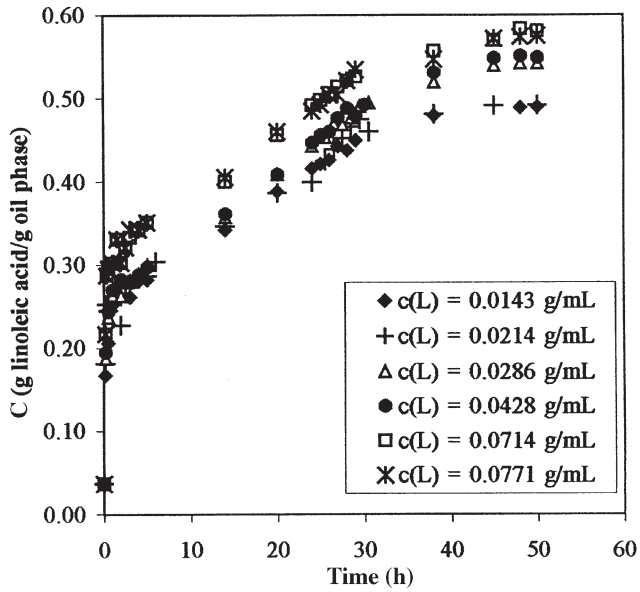

FIG. 2. Concentration of linoleic acid vs. time for different enzyme concentrations. The symbol c(L) indicates the amount of enzyme per mL of CO_2-free reaction mixture. The reaction was carried out in a high-pressure batch stirred tank reactor (HP BSTR) at 50°C, 200, bar for 50 h. SC CO_2 was used as a reaction medium. For abbreviation see Figure 1.

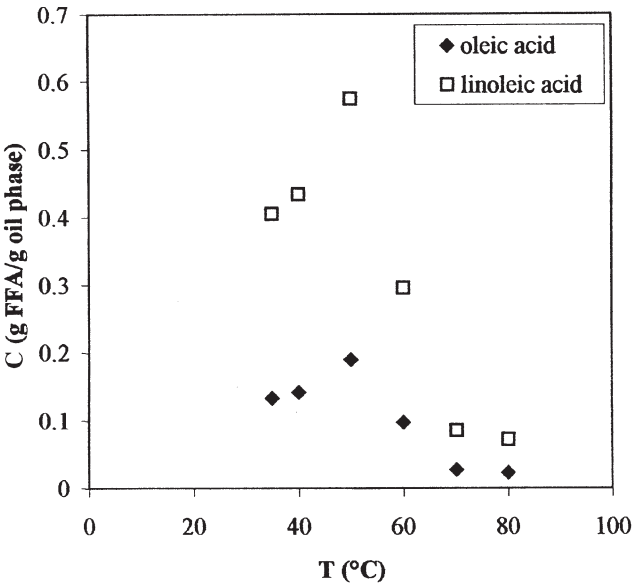

FIG. 3. Influence of temperature on the hydrolysis of sunflower oil in SC CO_2 at 200 bar. The reaction was catalyzed by Lipolase 100T and stirred at 600 rpm. The concentration of FFA after 48 h is presented. For abbreviations see Figure 1.

(ii) Effect of temperature and pressure on the conversion. Two effects are joined during an increase in reaction temperature: The reaction rate increases with higher temperature, and enzyme activation/deactivation occurs. However, where SCF are used as reaction media, the optimal temperature also depends on the operational pressure. With changes in pressure and temperature, the transport properties of SCF may change significantly, resulting in changes in the reaction rates.

For the hydrolysis of sunflower oil, the influence of temperature on the initial reaction rates as well as on yield after 48 h was studied.

The FFA concentration after 48 h increased with temperature (35–50°C) at 200 bar (Fig. 3), whereas with a further temperature rise it decreased. The highest degree of hydrolysis in SC CO_2 at 200 bar was at 50°C, which overlaps with the results of our study on lipase stability in this medium. The optimal pressure at 50°C for this reaction was 200 bar (Table 1). With higher (300 bar) and lower (100 bar) pressures, the concentration of both acids decreased slightly.

(iii) Influence of pH and buffer/oil ratio on the conversion. In aqueous environments, enzymatic activity is sensitive to the pH of the bulk solution. Even if one does not intend to study the pH dependence of an enzyme-catalyzed reaction, attention still must be given to the choice of pH (15). Therefore, the lipase-catalyzed hydrolysis of sunflower oil in SC CO_2 was performed in phosphate buffer solutions of different pH values. At optimal conditions for the aforementioned reaction (50°C and 200 bar), the optimal pH value was determined. The results are presented in Table 2. As expected (16), the optimal pH for hydrolysis of sunflower oil with Lipolase 100T was pH 7. At lower and higher pH values, reaction rates were lower. With pH values far removed from the optimal pH, reaction rates would be expected to decrease even more. Under these circumstances, the forces stabilizing the native protein conformation may be so disturbed that denaturation occurs. In this situation, we cannot expect the normal enzymatic activity to return quickly, if at all, in a practical time scale if the pH is restored to its optimal or near-optimal value (17).

Another parameter that also could influence the degree of hydrolysis is the substrate ratio. For this reason, the oil/buffer ratio was also optimized. As can be seen from Figure 4, the highest reaction rate at optimal conditions was achieved at a

TABLE 1
Concentration of FFA in HP BSTR After 48 h at 50°C and Different Pressures[a]

	100 bar	200 bar	300 bar
Linoleic acid (g linoleic acid/g oil phase)			
± 0.002[b]	0.557	0.586	0.549
Oleic acid (g oleic acid/g oil phase)			
± 0.002[b]	0.185	0.193	0.182

[a]HP BSTR, high-pressure batch stirred tank reactor.
[b]± 0.002 means SD.

TABLE 2
Dependence of FFA (concentrations) on pH in HP BSTR After 48 h at 50°C, 200 bar, and Enzyme Concentration of 0.0714 g Enzyme/mL of CO_2-Free Reaction Mixture[a]

	pH = 6	pH = 7	pH = 8
Linoleic acid (g linoleic acid/g oil phase)			
± 0.002[b]	0.415	0.586	0.545
Oleic acid (g oleic acid/g oil phase)			
± 0.002[b]	0.136	0.193	0.182

[a]For abbreviation see Table 1.
[b]± 0.002 means SD.

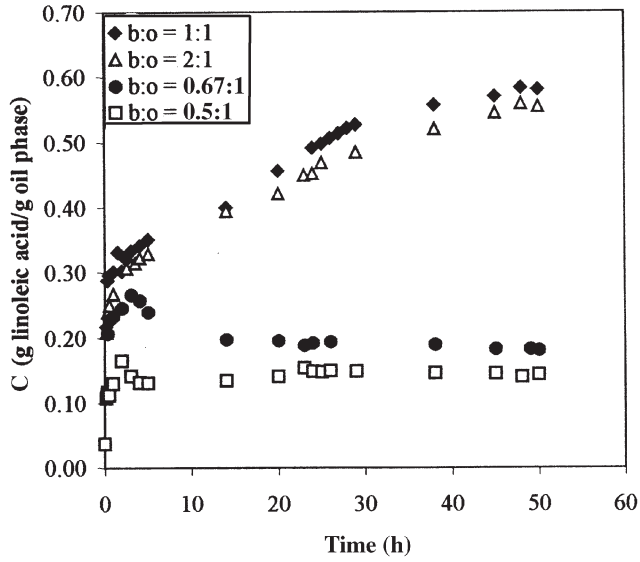

FIG. 4. Dependence of the free linoleic acid concentration buffer/oil ratio in HP BSTR at 50°C, 200 bar, pH = 7, at an enzyme concentration of 0.0714 g enzyme/mL of CO_2-free reaction mixture. The symbols b:o indicate the buffer/oil ratios (w/w) in the reaction mixture.

buffer/oil ratio 1:1 (w/w). At buffer/oil ratios of 0.5:1 and 0.67:1 (w/w), a much lower degree of hydrolysis was observed. It is not clear whether this effect is the result of the impact of the ratio of water and oil on the degree of emulsification of the oil or some other interaction between catalyst, water, and lipid.

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