Enzymatic Fatty Ester Synthesis

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Fatty ester synthesis with immobilized 1,3-specific lipase from *Mucor miehei* is described. 1,2-Isopropylidene glycerol produced by condensation of glycerol with acetone was esterified with oleic acid in the presence of a *Mucor miehei* lipase (Lipozyme™) to obtain 1,2-isopropylidene-3-oleoyl glycerol. The effects of various process parameters (temperature and pressure) and various ratios (enzyme/substrate) have been investigated to determine optimal conditions for the esterification process. The highest conversion of oleic acid (80% w/w) was obtained at 55°C and 0.057 bar, while the optimal addition of lipase to substrate was determined to be 0.096 g per gram of reaction mixture. The esterification can be modeled successfully as a reverse second-order reaction. Thermodynamic properties of the reaction system at 55°C and 0.057 bar also were determined. Activation energy was 20.82 kJ/mole, entropy of activation −0,26 kJ/(K mole) and free energy of activation was 103.32 kJ/mole.

KEY WORDS: Batch stirred tank reactor, biotechnology, ester synthesis, 1,2-isopropylidene-3-oleoyl glycerol, lipase, *Mucor miehei*, reaction kinetics, reaction thermodynamics.

Various fatty acid esters of glycerol may undergo conventional acid- or base-catalyzed reactions such as esterification, glycerolysis and hydrolysis (1–4). Recently, the use of enzymes to catalyze these reactions has become a much more promising method for fatty ester production (5–8). Enzyme-catalyzed reactions are superior to conventional chemical methods owing to mild reaction conditions, high catalytic efficiency and the inherent selectivity of natural catalysts (9), which results in much purer products. Lipases belong to the enzymatic class of hydrolases and are convenient enzymes for hydrolysis and formation of esters. Dependent on their activity towards the fatty acids in glycerides, lipases are classified as nonspecific or 1,3-specific.

Immobilization of enzymes usually leads to increased thermal stability. Diffusion of the reactants to the enzyme molecule affects the activity of the immobilized enzyme and can be facilitated at a high reaction temperature. However, high temperatures must be avoided because of potential enzyme denaturation (10).

In the case of nonimmobilized lipase application, the enzymatic reaction takes place in an emulsion system at the phase boundary between lipid and water where the lipase accumulates. Reaction with immobilized lipase (Lipozyme™) can be carried out in a nonemulsion system, because the immobilization support surface replaces the water-lipid interface. Since the immobilization support (macroporous anion exchange resin) enables the enzyme to retain the water essential for its appropriate active conformation, the water formed during the reaction is allowed to be removed from the reaction system. Reaction yields are reportedly higher by the addition of some water to the reaction system where Lipozyme™ is employed as a catalyst (11,12).

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The present paper reports on the method of preparation of 1,2-isopropylidene-3-oleoyl glycerol by using "blocked" glycerol (13). If desired, 1,2-isopropylidene-3-oleoyl glycerol can be transformed to 1-monooleoyl glycerol by acid treatment. 1-Monoacyl glycerols are widely used as emulsifiers in food, pharmaceutical and other applications (14). As a catalyst, 1,3-specific immobilized lipase from *Mucor miehei* (Lipozyme) was applied. Owing to a specially chosen immobilization support (macroporous anion exchange resin), the synthesis can be carried out either with or without a solvent (15). In the present study our goal was to investigate the process parameters for enzyme-catalyzed esterification of 1,2-isopropylidene glycerol with oleic acid in a solvent-free system. Some kinetic parameters and thermodynamic properties of the above-mentioned esterification process will be reported as well.

MATERIALS AND METHODS

Materials. Lipase (Lipozyme) was a generous gift from Novo Nordisk (Copenhagen, Denmark). Petroleum ether, sulphuric acid, toluene and acetone were p.a. grade from Kemika (Zagreb, Yugoslavia). Diethyl ether was obtained from Lek (Ljubljana, Yugoslavia) and oleic acid from Merck (Darmstadt, Germany); glycerol was purchased as a technical product of 95% w/w purity from Henkel-Zlatorog (Maribor, Yugoslavia).

Synthesis of 1,2-isopropylidene glycerol. 1,2-Isopropylidene glycerol was produced by acid-catalyzed condensation of glycerol with acetone (Fig. 1) in a 2-L stirred tank reactor with petroleum ether as a solvent. Water produced during the reaction was successfully removed from the system by azeotropic distillation. After completing the reaction, the product was distilled under vacuum.

Enzyme-catalyzed esterification of 1,2-isopropylidene glycerol. This reaction (Fig. 2) was performed in a 100-mL round-bottom flask connected to a vacuum pump in order to remove the co-produced water from the system and thereby shift the equilibrium towards ester formation. The desired quantity of Lipozyme was added to the thermostated equimolar mixture of 1,2-isopropylidene glycerol and oleic acid. The temperature and the pressure were kept constant. The reaction progress was followed by free fatty acid titration. The activity of lipase used in our experiments was 23 BIU/g. One BIU is defined as 1 mole of

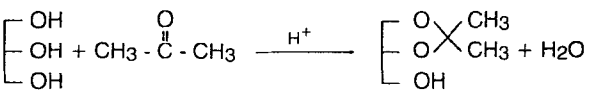

FIG. 1. 1,2-Isopropylidene glycerol process.

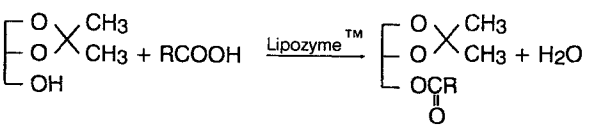

FIG. 2. Esterification process.

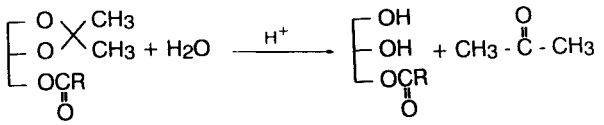

FIG. 3. Hydrolysis process.

palmitic acid incorporated in triolein per minute at standard conditions (15).

At the end of the reaction, water and diethyl ether were added and the enzyme was filtered off. The aqueous phase was extracted with diethyl ether, and combined organic layers were subsequently washed with 0.5 M NaOH. The obtained 1,2-isopropylidene-3-oleoyl glycerol can be hydrolyzed with concentrated hydrochloric acid in cold diethyl ether solution to obtain monoacyl glycerol by splitting out the acetone (Fig. 3). The background for the synthesis of 1,2-isopropylidene glycerol and 1,2-isopropylidene-3-oleoyl glycerol can be found in a patent by Godtfredsen (13).

RESULTS AND DISCUSSION

The purity of 1,2-isopropylidene glycerol was estimated by gas chromatography followed by mass spectrometry. It was found to be at least 98% w/w. A Hewlett-Packard 5840A gas chromatograph was connected with a Hewlett-Packard 5985 mass spectrometer (Palo Alto). The conditions were: column, SPB-5 fused silica capillary (30 m × 0.32 mm i.d.); program, 40–250°C at 10°C/min; injection temperature, 190°C; transfer line temperature, 250°C. The mass spectra were recorded within the interval from 41 to 250 m/e. To determine optimal process parameters, the esterification of 1,2-isopropylidene glycerol with oleic acid was carried out at three different pressures—1 bar, 0.057 bar and 0.032 bar in the temperature interval from 10°C to 70°C. The results are shown in Figure 4.

It is evident from Figure 5 that the optimal conditions for the esterification that lead to 80% w/w equilibrium conversion of oleic acid are 55°C and 0.057 bar. At 1 bar the water generated during the reaction was not removed from the system so as to push the equilibrium towards ester formation, which results in lower equilibrium conversion of oleic acid. At the pressure of 0.032 bar the reaction yield is surprisingly lower in comparison with that at 0.057 bar. At 0.032 bar much water is removed from the system, which may be reflected in lower conversion due to decreased enzyme activity at low water content (11,16). The same conclusions could be made for the results obtained at 40°C. At 25°C we have obtained the greatest conversion at 0.032 bar when compared to those at 0.057 bar and 1 bar. This may be explained by the fact that more water is removed from the system at lower pressure than at higher pressure, thus shifting the equilibrium towards ester formation. Moreover, because of lower vapor pressure of water at lower temperatures, it may be assumed that at 25°C not as much water is removed from the system as to decrease the enzyme activity as might be the case at higher temperatures. It is expected that elevated temperatures result in higher reaction yields. On the other hand, as the deactivation rate of the enzyme increases with temperature, reaction yields and conversions are found to be lower at 70°C when compared with those at 55°C. Moreover, higher conversion is obtained at 1 bar than at 0.032 and 0.057 bar. Lower conversions at diminished pressures at 70°C might be explained by more efficient removal of water and, therefore, decreased enzyme activity.

It is expected that a greater amount of enzyme results in a higher reaction rate or in a shorter time in which equilibrium conversion is reached. This effect was investigated through several experiments. It was found that the maximum conversion of oleic acid (80%, w/w) can already be achieved after approximately 350 min by the addition of 0.096 g lipase per gram of reaction mixture or 2.2 BIU/g (17).

The de-blocking of ketal ester by means of hydrolysis (Fig. 3) with diluted HCl to obtain monoacyl glycerol was found to be a temperature-dependent process. However, pure monoacyl glycerol was separated from unhydrolyzed 1,2-isopropylidene-3-oleoyl glycerol on a silica gel column by the adsorption chromatographic technique (18) and was identified by thin-layer chromatography (19). Our future efforts will, therefore, focus on the optimization of the above-mentioned de-blocking process to obtain monoacyl glycerol as pure as possible.

Determination of reaction order and rate constants. By considering the esterification to be a reverse second-order reaction of equal initial concentrations, the general form of the rate equation for consumption of oleic acid is (20):

$$dC_a/dt = k_1C_a^2 - k_2C_c^2 \quad [1]$$

where C_a is the concentration of oleic acid (mole/L) and C_c is the concentration of 1,2-isopropylidene-3-oleoyl glycerol (mole/L).

Applying the conversion of oleic acid (X_a) instead of its concentration, the solution of the corresponding differential equation becomes:

$$\text{Ln}\ ((X_e - X_a(2X_e - 1))/(X_e - X_a)) = 2k_1(1/X_e - 1)C_{a0}t \quad [2]$$

where X_e is an equilibrium conversion of oleic acid, C_{a0} is the initial oleic acid concentration (mole/L), t is the reaction time (s), and k_1 is the rate constant for esterification [L/(mole s)]. Considering the reaction in its steady state, the correlation between k_1 and the rate constant for the reverse reaction, k_2, is:

$$k_2 = k_1(1 - X_e)^2/X_e^2 \quad [3]$$

From the slope of the straight line obtained by plotting the left side of the equation 2 *vs.* time (Fig. 6), the rate constants k_1 and k_2 were determined at 55°C to be 2.40×10^{-4} L/(mole s) and 1.33×10^{-5} L/(mole s), respectively. The equilibrium constant calculated from the rate constants k_1 and k_2 was found to be 18.04, which seems to match well with the constant calculated from the experimental steady state data by the following equation (20):

$$K_x = X_{ce}X_{de}/X_{ae}X_{be} \quad [4]$$

where X_{ae} and X_{be} are mole fractions of reactants in equilibrium, while X_{ce} and X_{de} are mole fractions of products in equilibrium. The equilibrium constant calculated in this way was 17.99.

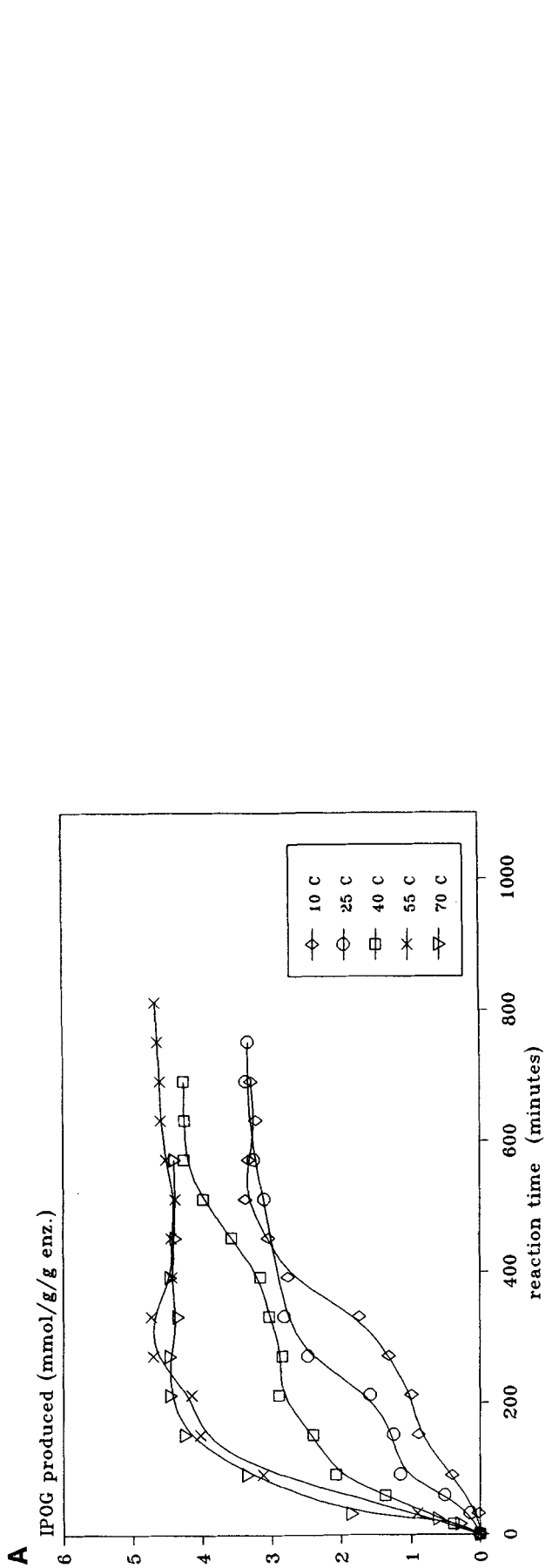

FIG. 4. Progress curves of esterification (a) at 1 bar, (b) at 0.057 bar, and (c) at 0.032 bar.

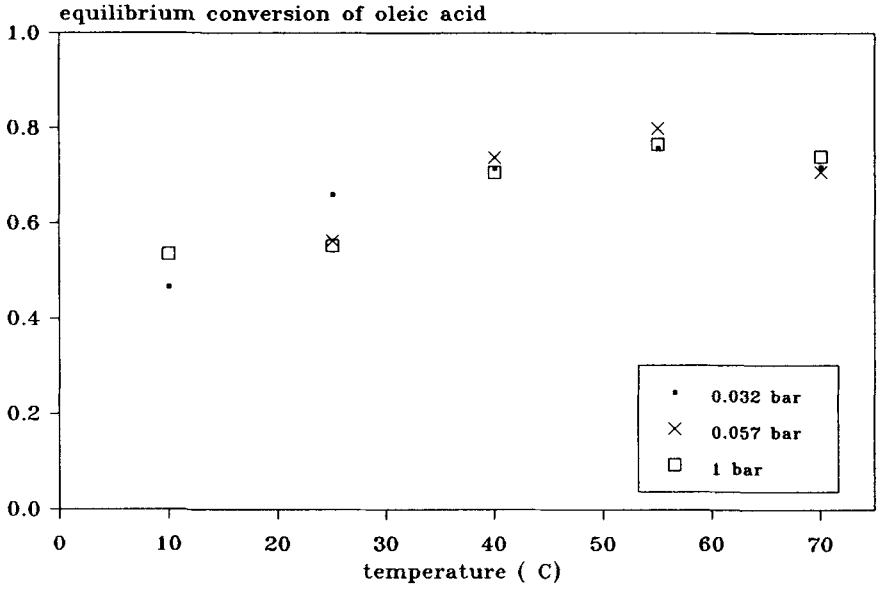

FIG. 5. Effect of temperature on equilibrium conversion of oleic acid at different total pressures of reaction system.

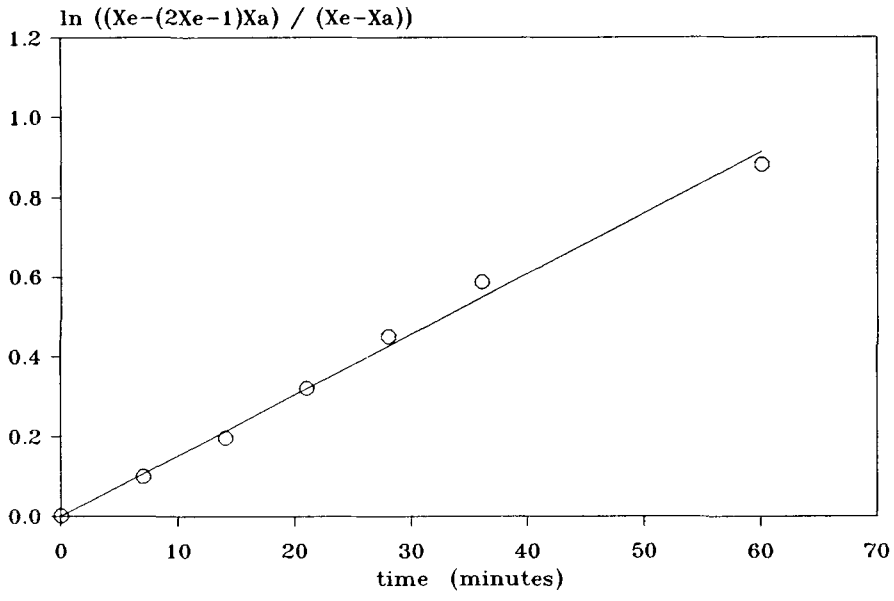

FIG. 6. Order of reaction and initial rate constants determination; reverse second-order interpretation.

Thermodynamic properties of the reaction system. Activation energies of the esterification process at 1 bar, 0.057 bar and 0.032 bar were determined by applying Arrhenius' law (21). Determining initial velocities at different temperatures and pressures from Figure 4, the Arrhenius plots (Fig. 7) can be constructed. On the basis of slopes of straight lines, the activation energies E_a, were calculated as 32.41 kJ/mole at 1 bar, 20.82 kJ/mole at 0.057 bar and 30.65 kJ/mole at 0.032 bar.

The enthalpies of activation, ΔH_a, were calculated at the same pressures and at 55°C by using the equation $\Delta H_a = E_a - RT$. They were found to be 29.68 kJ/mole, 18.09 kJ/mole and 27.92 kJ/mole, respectively. The free energy of activation, ΔG_a, and the entropy of activation, ΔS_a, at 55°C and 0.057 bar were calculated as in Laidler (20) and determined to be 103.32 kJ/mole and −0.26 kJ/(Kmole), respectively.

These results clearly indicate that the minimal energy and enthalpy of activation for esterification were required as the reaction was carried out at 0.057 bar. The energy

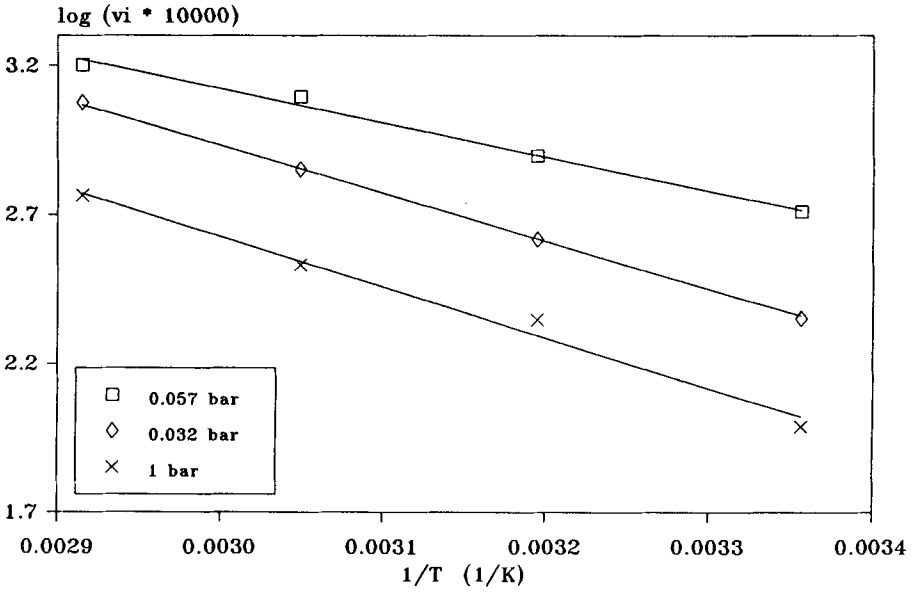

FIG. 7. Determination of activation energy depending on total pressure of reaction system.

of activation was found to be temperature independent in the interval from 10°C to 70°C. The negative entropy of activation is consistent with an increase in polarity as the activated complex enzyme/substrate is formed (20).

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