Enzymatic Acylglycerol Synthesis in a Membrane Bioreactor

A. van der Padt*, M.J. Edema, J.J.W. Sewalt and K. van 't Riet
Wageningen Agricultural University, Food and Bioprocessengineering Group, Dept. of Food Science, Biotechnion, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

Lipases can catalyze the esterification reaction in a two-phase system. The *Candida rugosa* lipase-catalyzed esterification of decanoic acid with glycerol is described in this work for an emulsion system and for a hydrophilic membrane bioreactor. The enzymatic activity is studied in relation to the interface area between the two phases, the enzyme load and the reactor volume. The initial rate per unit interface area, the interfacial activity, is roughly equal for both systems indicating that the cellulose membrane does not hinder the esterification. Because the interfacial activities are equal, the volumetric activity of a membrane system is only specific area related, so a hollow fiber membrane device is preferable. The activity is also a function of the enzyme load. The optimum load in a hydrophilic membrane reactor is one to three times the amount of a monolayer, while in an emulsion system several times this amount. This could indicate that in the emulsion system the adsorption is in a dynamic state while at the membrane surface the adsorption reached its equilibrium state.

This paper deals with lipase-catalyzed esterification. The general esterification reaction is that of an acid group (often a fatty acid) with an alcohol group with the formation of the ester and a molecule of water. Lipases are able to catalyze esterification reactions of fatty acids with alcohols, producing fats and oils and even fine chemicals (chiral compounds, epoxy esters). Sometimes the alcohol and the fatty acid are mutually soluble; usually, however, a two-phase system is obtained. This article will be restricted to the two-phase system. In case of a two-phase system, the reaction occurs at the interface in the presence of the enzyme (Fig. 1). The industrial interest in esterification and hydrolysis is growing very fast. Therefore research on the process engineering of esterifying systems should point out whether or not these systems are economically viable.

In the literature, enzymatic hydrolysis, interesterification and esterification are described. Several publications describe the hydrolyses of oils and fats for the production of bulk fatty acids or special fatty acids (e.g. ricinoleic acid) in an emulsion system (1) or in a membrane system (2,3). The fat and oil interesterification experiments are mostly concerned with an immobilized lipase on lab scale (4) or on pilot plant scale (5). Work is also done on the synthesis of oils and fats by esterification starting with the basic compounds using an enzyme as catalyst (6-12). These lipase catalyzed reactions at moderate temperature and low water activity are different from the method as presented by Zaks and Klibanov (13); their experiments showed that enzymes can also be active in organic media with an extremely low water content (approximately 0.02%), and sometimes also at high temperatures (up to 100°C).

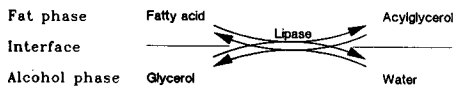

FIG. 1. The fatty acid triacylglycerol equilibrium.

*To whom correspondence should be addressed.

The literature (Table 1) describes three lab-scale esterifying systems for two-phase reaction media:

(i) The use of a solvent to change the two-phase system in a one-phase system: a solvent is chosen in which both the fatty acid and the alcohol are dissolved e.g. acetone, hexane or methyl isobutyl ketone (MIBK). The obtained enzyme activity is not only solvent dependent, but also a function of the nature of the substrate and its concentration as well as of the enzyme source. For example Bell *et al.* (6) showed acylglycerol synthesis in acetone catalyzed by *Rhizopus arrhizus* lipase, while Miller and coworkers (7) found almost no activity for the synthesis of propyl myristate in acetone catalyzed by *Mucor miehei* lipase. Since most lipases are known to be active at an interface only, the enzyme must be immobilized, creating a solvent/immobilization carrier interface. Bell *et al.* (6) used the mycelium of the lipase-producing *Rhizopus Arrhizus* itself, while Miller *et al.* (7) used a commercially available immobilized lipase.

(ii) Two-phase emulsion system: the alcohol phase, the fatty acid phase and the lipase, either on small particles or "free," are mixed and thus an emulsion is obtained. The emulsion interface is created by energy input and the emulsifying qualities of the lipase itself. Sometimes an emulsifier is added (8). The lipase adsorbs to the interface.

(iii) The two-phase membrane system: the two phases are kept separated by a membrane—this membrane is used as the immobilization surface. One of the substrates diffuses through the membrane towards the interface between the two phases where the enzyme is immobilized.

The lipases are too costly to be used for a single batch conversion only. Therefore, reuse of the enzyme determines the success of an enzymatic synthesis in case of an industrial process. Two methods for reuse of the enzyme in two-phase emulsion systems are published. Firstly, for the hydrolysis of an oil, it was shown that after centrifugation of the emulsion, the recovered interfacial layer contained about 80% of the initial activity (14). Secondly, immobilization of the biocatalyst by entrapment in a gel as immobilization carrier or adsorption or covalent binding on grains the lipase could be recovered and used for several batches (15). Another method for reuse of the enzyme is immobilization at the interface between the oil and alcohol phase in a membrane bioreactor. An additional advantage of the membrane reactor is the *in situ* separation of the fatty acid phase and the alcohol phase.

Hoq *et al.* (11,12) presented a hydrophobic membrane system for hydrolysis as well as for ester synthesis. The lipase is immobilized at the water-glycerol side of the membrane. A complication appeared to be that lipase does only partly adsorb at the membrane surface at high glycerol contents. In that case an enzyme-glycerol-water solution is circulated at one side of the membrane and high quantities of enzyme are required. Pronk (2) developed an enzymatic membrane system for the hydrolysis

TABLE 1

The Esterifying Systems in Literature

System	Fatty acid	Alcohol	Lipase	Solvent	Reference
One-phase	palmitic	octanol	*Rhizopus Arrhizus*	diisopropyl ether	Bell *et al.* (6)
	C_4-C_{10}	octanol	*Mucor miehei*	hexane, MIBK, acetone among others	Miller *et al.* (7)
	oleic	glycerol	*Rhizopus Arrhizus*	acetone	Bell *et al.* (6)
Emulsion	oleic among others	glycerol among others	*Aspergillus niger*		Tsujisaka *et al.* (8) and Okumura *et al.* (9)
			Rhizopus delemar		
			Geotrichum		
			Candidum		
			Penicillum cyclopium		
	oleic	glycerol	*Aspergillus niger*		Linfield *et al.* (10)
			Pseudomonas sp.		
			Mucor pusiilus		
			Geotrichum candidum		
			Rhizophus delemar		
Membrane	oleic	glycerol	*Candida rugosa*		Hoq *et al.* (11,12)
			Mucor miehei among others		

of soybean oil catalyzed by lipase of *Candida rugosa.* Here the lipase is immobilized at the oil side of the membrane and because the membrane is impermeable for the enzyme, the enzyme cannot dissolve in the glycerol-water phase. The hydrophilic membrane that is used can withstand transmembrane pressures larger than 10^5 Nm^{-2}, without leakage of the oil phase into the glycerol-water phase, which has distinct operation advantages.

It is not yet possible to determine which membrane system should be preferred for use in a large-scale process. More data are needed, particularly about the activity of an esterifying hydrophilic membrane system. The objective of this study is to show the relationship between the enzymatic activity of a membrane bioreactor and a similar emulsion system.

Theory: Immobilization of a biocatalyst can lead to an activity change not only caused by the immobilization itself, but also caused by diffusional limitations in the immobilized biocatalyst system. The measured apparent activity of the immobilized system should be compared with the activity of the free-enzyme system (16). For a batch reactor, the activity equals to the initial substrate removal rate r_i (mole/m^3/s):

$$r_i = (-\ d\ C_S/dt)_{t=0} \qquad [1]$$

where C_S is the substrate concentration (mole/m^3) and t is the time (s). Dealing with an enzymatic heterogeneous equilibrium reaction, three standard activities can be calculated: the volumetric initial rate, the interfacial initial rate and the enzymatic initial rate.

The volumetric initial rate r_{iV} (mole/m^3/s) is based on the total reactor volume, and is a measure given for the batch system volumetric efficiency. By nature of its definition, it is given by:

$$r_{iV} = r_i \qquad [2]$$

The enzymatic initial rate r_{iE} (mole/s/kg) is based on the enzyme present, and indicates the enzyme activity:

$$r_{iE} = r_i/C_E \qquad [3]$$

where C_E is the enzyme concentration (kg/m^3).

The interfacial initial rate r_{iA} (mole/s/m^2) is based on the interfacial area, and is a measure for the activity on the interface:

$$r_{iA} = r_i/A \qquad [4]$$

where A is the specific area (m^2/m^3).

These three activities should point out whether or not the membrane reactor can compete with an emulsion system. The activity shows the initial rate of the reaction indicating that the higher the activity the sooner the equilibrium state is reached. The equilibrium value is

thermodynamically determined so an emulsion experiment ends up with the same concentration as the membrane system.

EXPERIMENTAL

Materials. The 97% pure fatty acid, decanoic acid, was produced by Unichema Chemie (FRG) for this project. Glycerol 99+% was obtained from Janssen (Belgium), hexane from Rathburg (UK), all other chemicals were gained from Merck (FRG). The *Candida rugosa* lipase (formerly name *Candida cylindracea*) was the enzyme Lipase-OF 360 (Meito Sangyo, Japan). The commercial available Andante membrane (Organon, Holland) was a hollow fiber device containing cellulose (cuprophane, Enka, FRG) fibers with a diameter of 0.2 mm and a wall thickness of 8 μm, the total area was 0.77 m^2, and the reactor volume was 150 mL.

Methods. The emulsion system was run in a thermostated (25°C) reactor of standard geometry (inner diameter = 0.1 m) with four baffles and a four-bladed turbine (diameter = 0.04 m). The reactor was filled with 500 mL decanoic acid in hexadecane (1:1, w/w) and 220 mL glycerol-water with a known water content. The two phases were mixed (960 rpm) for 30 min. After that, the crude enzyme (2 grams) diluted in 30 mL glycerol-water was added. The initial rate was measured after 20 min to allow the emulsion to stabilize.

The oil and alcohol circuits of the membrane system were filled. The membrane system had an oil-phase volume of 140 ± 10 mL decanoic acid in hexadecane (1:1, w/w) circulating through the inner fiber side (2 L/hr). At the shell side, 100 mL glycerol-water phase was recycled (3 L/hr). To immobilize the crude lipase, 1 gram was diluted in water, followed by centrifugation (300 s, 30,000 rpm) to remove the cell debris. During the centrifugation step, no activity loss occurred. The clear liquid was dispersed in the oil phase, and over a 3-hr period, ultrafiltrated from the inner fiber side towards the shell side, thus immobilizing the lipase on the inner fiber side (2). When the system reached equilibrium, the oil phase was refreshed, thereby removing the surplus of free enzyme. Then the initial rate could be measured. The initial rate was measured for a number of glycerol concentrations at 25°C. The glycerol concentration was determined using a Pleuger refractometer.

Fat-phase analysis was done using a Carlo Erba gas chromatograph with a cold on-column injection system. Conditions: 5 meter CP-sil-5CB (Chrompack, Holland) capillary column with injection temperature: 80°C, cooling 10 sec, oven temperature: 1 min isotherm, 2°C/min up to 320°C; carrier: He flow: 2.6 mL/min, detection: FID 370°C, H_2 flow: 19 mL/min, and air flow: 107 mL/min.

The droplet size was measured under the microscope. For each emulsion, four samples were taken and placed in a Hemacytometer (improved Neubauer). The Sauter mean diameter d_{32} (m) was calculated for four emulsions with:

$$d_{32} = \Sigma (n_i \cdot d_i^3)/\Sigma (n_i \cdot d_i^2) \qquad [5]$$

where n_i is the number of droplets with diameter d_i (m).

The specific area A (m^2/m^3) is given by:

$$A = 6.\Phi/d_{32} \qquad [6]$$

where Φ is the volume fraction of disperse phase (m^3 of disperse phase/m^3 of reactor volume).

RESULTS AND DISCUSSION

The esterification was measured in the emulsion system as well as in the membrane system. The initial rate and the equilibrium values were determined at different glycerol concentrations.

An example of a typical run in both systems is shown in Figure 2. In the membrane system the fat phase is replaced when equilibrium is reached. The enzyme stays active, even after three batches. Each subsequent batch ends up with a higher free fatty acid equilibrium concentration, due to the reduced glycerol concentration caused by the water produced. The equilibrium concentrations for a number of emulsion and membrane batches are shown in Figure 3, as well as the values given by Tsujisaka *et al.* (8) for a glycerol/oleic acid emulsion system. The equilibrium values of Tsujisaka for oleic acid are nearly the same as our values for capric acid. Apparently the equilibrium concentration is mainly dependent on the water concentration for these two cases. At a glycerol concentration higher than 0.65 mole/mole, the measured equilibrium values decrease with increasing glycerol concentrations. Most probably this is an apparent decrease because the thermodynamical equilibrium might not be reached because of the inactivation of lipase by glycerol. During the reaction, the enzyme is inactivated

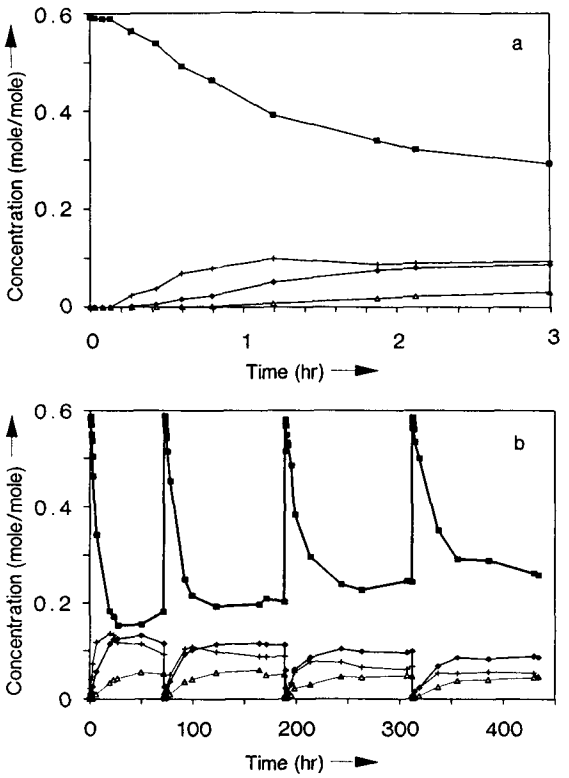

FIG. 2. The ester synthesis in an emulsion system (a) and four batches of ester synthesis in a membrane system (b). Solid square = capric acid, + mono-; open diamond = di-; and open triangle = tricaprinate.

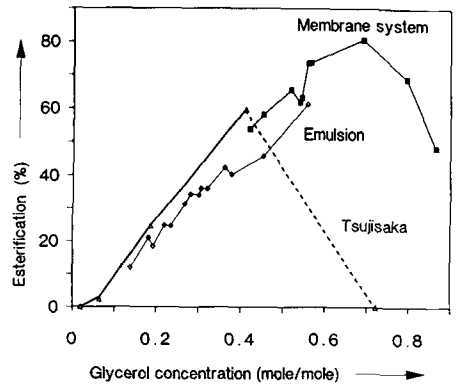

FIG. 3. Measured fatty acid equilibrium concentrations at various glycerol concentrations for the emulsion system and the membrane system and literature values (8).

and the inactivation is complete before the equilibrium value can be reached. The inactivation of enzymes in low-water activity solvents is correlated with the log P value (17). Log P is defined as the logarithm of the partition coefficient in a standard octanol water two-phase system. For glycerol, the calculated log P is negative, resulting in complete inactivation in pure glycerol. The glycerol influence can be reduced by using a solvent, such as THF or 2-pentanone (6,13) but in that case a one-phase system is obtained.

The initial rate is measured for several membrane and emulsion experiments at a variety of the initial glycerol concentrations. The volumetric initial rate is presented in Figure 4a. The decrease of activity is indeed observed at high glycerol concentrations. If this is due to inactivation of the enzyme instead of a kinetic effect of the glycerol, then the membrane system has lost its activity. This is tested for a high glycerol membrane system without activity by diluting the glycerol phase at the start of a following batch. Indeed no activity is measured.

To calculate the interfacial initial rate, the specific area A (m^2/m^3) of the emulsion must be known. The Sauter mean diameter d_{32} for this alcohol in oil emulsion is measured as 0.10 ± 0.02 mm, resulting in a specific area $A = 18 \cdot 10^3$ m^2/m^3. The specific area of the membrane is calculated as the ratio of the membrane surface area and the reactor volume resulting in $A = 5 \cdot 10^3$ m^2/m^3. The interfacial initial rates are given in Figure 4b. The rates of both systems are of the same order, pointing out that, within the range of experimental accuracy of the specific area measurement, the cellulose membrane does not affect the interfacial activity. Optimum esterification takes place where both the equilibrium value as well as the initial rate are high. At glycerol concentrations between 0.3 to 0.5 mole/mole glycerol, the interfacial initial rate in the membrane system exceeds the emulsion system, while the emulsion system is twice as active at higher glycerol concentrations. The optimum in the membrane activity could be due to the difference in enzyme load at the actual interface, as further discussed below. The very low activity at high glycerol concentrations could be caused by inactivation. As stated above, the inactivation is a function of the glycerol concentration. Assuming that this inactivation is not instantaneous and taking into account that the membrane system is

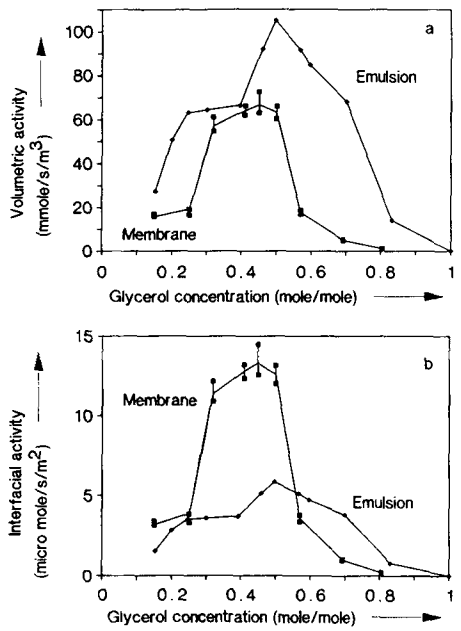

FIG. 4. The volumetric activity (a) and the interfacial activity (b) versus the glycerol concentration for both the emulsion system and the membrane reactor.

measured after approximately 70 hr using the second oil-batch data (Fig. 2) instead of the 0.4 hr in the emulsion system, the decrease of the activity in the membrane system is indeed likely to be due to the inactivation.

Dealing with an interface reaction, the activity is not only a function of the enzyme activity but also of the enzyme coverage of the interface. To determine the enzyme activity, the enzyme load must be optimized to make sure all of the enzyme participates in the reaction, so that the enzymatic activity is not underestimated. Two regions are expected: firstly an increasing activity with the enzyme load, in the case that there is still unoccupied interface left, so the interfacial activity is proportional to the enzyme load; secondly, once the interface is fully occupied with one or a number of active enzyme layers, a constant activity is obtained, even when the enzyme load is increased. Now the activity is proportional to the specific area. The change from enzyme limitation to interface limitation should mark the point where the optimum number of enzyme layers is formed. Assuming that a monolayer is the optimum enzyme occupancy, the load could be calculated based on the Stokes radius. O'Connor and Bailey (1) estimate that, in this case, an interface can contain 1.6 mg pure pancreatic lipase/m^2. This corresponds to 31 mg crude *Candida rugosa* (5% pure lipase).

The occupation of the interface in the emulsion system is studied measuring the interfacial initial rate at different enzyme loads (Fig. 5). Indeed two regions can be distinguished: firstly up to 170 mg crude lipase/m^2, the rate is enzyme limited; secondly, for loads higher than 170 mg crude lipase/m^2, the rate is interface limited. The slight increase in rate could be due to the increase in the interfacial area as a result of the emulsifying qualities of the lipase, while the interfacial area was assumed constant for the calculations.

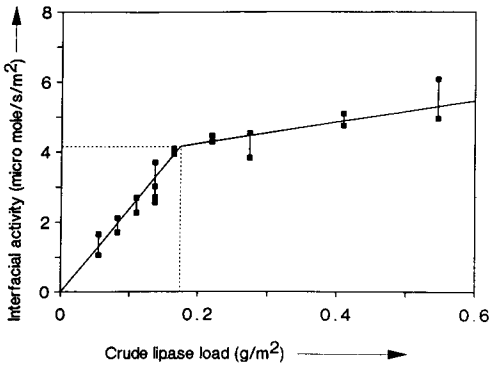

FIG. 5. The interfacial activity versus the enzyme load in the emulsion system.

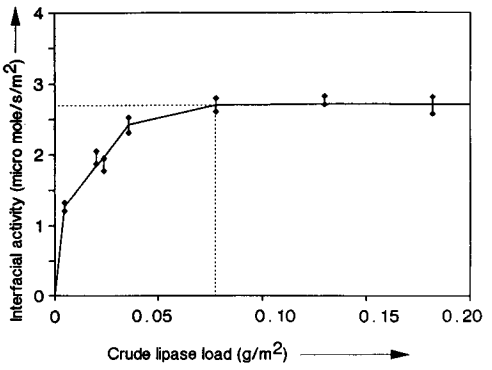

FIG. 6. The interfacial activity in a membrane system versus the enzyme load.

When assumed that only lipase adsorbs, the optimum load in the emulsion system of 170 mg crude lipase/m^2 equals five times the value calculated above for a monolayer coverage. This relative high load could indicate three different mechanisms. A possibility is that an active multilayer is formed. This assumption is not very likely, because the enzymatic activity in the membrane system is higher than in the emulsion system, even though the optimum enzyme load is lower (see below). Another possibility is that the excess of lipase is necessary because lipase only partly adsorbs at the fat/water-glycerol interface (18). Then a certain bulk concentration is needed to obtain an optimum enzyme layer at the emulsion interface. Moreover, due to the continuous dispersion and coalescence process that takes place in the emulsion, the surface is continuously created and destroyed. In this case the adsorption is still in a dynamic state, and takes place at the continuous created and thus unoccupied interface. Now both the dispersion rate and the adsorption rate are of great importance. The adsorption rate is enhanced when a surplus of lipase is present in the bulk. Because the enzyme load is calculated as the quotient of the enzyme added and the calculated specific area, the actual enzyme load should be corrected for the enzyme present in the bulk, which results in a higher enzymatic activity than the calculated apparent enzymatic activity. Unfortunately the enzyme concentration in the bulk cannot be measured, and moreover this surplus is necessary to obtain the optimum enzymatic activity, therefore the optimum load is defined as the quoteint of the crude lipase added and the interfacial area. Up to the optimum load, the enzymatic activity is constant, and in this region the enzymatic activity r_{iE} = 25 mmole/s/kg is characteristic for this system.

This experiment is repeated using a membrane reactor, and the results are given in Figure 6. Again, two regions are found, but now the optimum load is reached for 75 mg crude lipase/m^2, which is only two to three times the calculated value needed for a monolayer of pure lipase. At this load the enzymatic activity is 3.5 mmole/s/kg, which is 1.4 times the activity measured in the emulsion system. This could indicate two independent mechanisms: firstly, the membrane may stabilize the enzyme so it keeps its activity during the first batch, while the free lipase in the emulsion inactivates rapidly in 0.57 mole/mole glycerol; secondly, during the immobilization, the enzyme will adsorb preferentially at the membrane surface (19). This can be explained by a mechanism by which, at the start, all proteins near the membrane surface adsorb, but all proteins with a lower adsorption energy are replaced in time by lipase with a higher adsorption energy, so a relative, high interfacial activity is obtained after immobilization. This is stressed by the nonlinearity of the enzyme-limited region (Fig. 6). The optimum enzymatic activities are presented in Table 2.

TABLE 2

The Activities for the Emulsion System, Membrane System, and Hoq's System (12) with the Temperature Corrected Activity Between Parentheses

	Emulsion system (this work)	Membrane system (this work)	Membrane system (Hoq (12))	
Membrane:		hydrophilic	hydrophobic	
Temperature:	25°C	25°C batch	40°C continuous	
Fatty acid:	capric acid	capric acid	oleic acid	
Glycerol:	0.57 mole/mole	0.57 mole/mole	0.87 mole/mole	
Enzyme:	*Candida rugosa*	*Candida rugosa*	*Mucor miehei*	
mmole/s/m^3	97	19	28	(11)
μmole/s/m^2	4.1	3.5	11.3	(4.5)
mmole/s/kg	25	35	0.8	(0.3)

In the literature only a hydrophobic membrane device is described for the enzymatic ester synthesis (11,12). The activities of this continuous system are represented in Table 2. There are three differences between the systems which influence the activity:

(i) A higher temperature gives a higher activity (Q_{10} = 2, (20)). The corrected values are placed in brackets.

(ii) Different lipase type—it is shown in literature (9,12) that other kinds of lipase give other activities. Hoq found no activity at all using *Candida rugosa* in the hydrophobic membrane reactor (12). This could be due to the high glycerol concentration of 0.87 mole/mole resulting in a rapid inactivation so no esterification is measured (Fig. 4).

(iii) Membrane material—the advantage of an asymmetrical hydrophobic membrane, which has a rough surface, is the high surface area on microscopical scale per square meter visible membrane, so apparently a high

interfacial activity is gained. Therefore it is not surprising that the interfacial activity of Hoq's system is higher. However, a disadvantage is the fact that the fat/alcohol interface is at the alcohol-phase side of the membrane. The enzyme is therefore immobilized on the alcohol side and so there will be a distribution of enzyme adsorbed and enzyme solubilized in the alcohol phase so a surplus of the enzyme should be added (12). Using a hydrophilic membrane, with the enzyme in the oil phase, the enzyme cannot dissolve in the alcohol phase, because it cannot pass the membrane. This phenomenon explains the forty-times higher enzymatic activity for the hydrophilic system.

Both membrane systems have advantages. The hydrophilic system has a higher enzymatic activity, so this system could be used dealing with expensive enzymes taking the low interfacial activity for granted. On the other hand, a hydrophobic unit could be used for bulk processes, using cheap lipases and high production flows. For industrial processing, the hydrophilic membrane system has the advantage of withstanding transmembrane pressures without leakage of the oil phase. For the development of an industrial process, the three activities have to be studied related to the operational costs.

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