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Lipase-Catalyzed Ethanolysis of Milk Fat with a Focus on Short-Chain Fatty Acid Selectivity

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Mixtures of fatty acid ethyl esters were produced by lipase-catalyzed ethanolysis of milk fat triglycerides. Three commercial immobilized lipases (Lipozyme TL, Lipozyme RM, and Novozym 435) were tested in different reaction conditions with the aim of maximizing the conversion of the short-chain fatty acid fraction of milk fat to flavor ethyl esters. The influence of the reactants molar ratio was investigated, as well as three different reaction media, that is, hexane, CO_2-expanded liquid (GXL), and the solvent-free mixture. Novozym 435 showed the highest activity in all conditions. This lipase also exhibited selectivity for short-chain fatty acids, which, at short reaction times, resulted in a product mixture richer in short-chain fatty acids than the original milk fat. The highest selectivities were obtained in hexane and in CO_2-expanded liquid fat, at low ethanol to fat ratios. Using dense CO_2 as the reaction cosolvent is attractive because it results in the largest short-chain fatty acid enrichment in the product mixture, while leaving no residues in the product.

KEYWORDS: Short-chain fatty acid; milk fat; lipase selectivity; ethanolysis; dense carbon dioxide; GXL

INTRODUCTION

Ethyl esters of short-chain fatty acids are commonly used as flavoring agents in the food industry. Each short-chain fatty acid ethyl ester (FAEE) has a particular flavor, and especially, butyric (C4), caproic (C6), and caprylic (C8) ethyl esters are valued for their fruity resemblance (*1*). Currently, these flavor esters are produced via chemical synthesis, fermentation, or extraction from plant materials (*2*). Food grade FAEEs can be synthesized by lipase-catalyzed transesterification of oils and fats with ethanol, also known as ethanolysis (*3*). Lipase selectivity is a feature of great interest when the purpose of the transesterification reaction is synthesizing structured lipids or selectively enriching the reaction products in a certain fatty acid type. For example, several researchers have used lipases that discriminate against polyunsaturated fatty acids in hydrolytic reactions (*3, 4*). Lipase *sn*-1,3 selectivity has also been applied for synthesizing structured triacylglycerols (*5*).

Milk fat is one of the few natural sources containing a significant amount of short and medium fatty acids (C4−C12), around 25% on a molar basis (*6*). The distribution of fatty acids in milk fat triglycerides (TGs) is nonrandom, as shown in **Table 1** (*6, 7*); the short-chain fatty acids are mostly esterified in the primary positions of glycerol. Considering this fatty acid distribution, either a *sn*-1,3 specific or a short-chain specific lipase can be advantageous for a preferential release of short-chain fatty acids from the TGs.

Lipase-catalyzed reactions can be performed in organic solvents or solvent-free media. Solvent-free systems, although more viscous, eliminate the need of subsequent solvent−product separation steps (*8*). The reaction environment affects kinetics, equilibrium conversion, and enzyme selectivity (*3*); thus, for each application, an optimal solvent system may exist. Many studies have compared lipase activity and selectivity (*8*−*11*) in different solvent media. Generally, lipase activity correlates well to the solvent polarity (*10*−*12*), although many factors play a synergic role in determining activity on a specific system. On

Table 1. Average Fatty Acid Positional Composition of Cow Milk Fat, in Mol%

		positional distribution[b]		
fatty acid	overall[a]	*sn*-1	*sn*-2	*sn*-3
butyric (C4)	9.6	0	0	100
caproic (C6)	2.9	0	7	93
caprylic (C8)	2.8	25	12	63
capric (C10)	3.6	17	27	56
lauric (C12)	5.4	42	53	5
myristic (C14)	15.4	29	52	19
palmitic (C16)	28.4	47	45	8
hexadecenoic (C16:1)	5.1	36	46	18
stearic (C18)	8.9	49	45	6
oleic (C18:1)	16.0	42	26	32
linoleic (C18:2)	1.8	23	47	31

[a] Adapted from ref *7*. [b] Adapted from ref *6*.

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the other hand, no general rules about the effect of solvents on the selectivity of lipases have been deduced (*12*).

A novel reaction solvent in which lipase reactions have been studied in recent years is supercritical carbon dioxide ($scCO_2$). $scCO_2$, comparable to organic solvents in terms of polarity, is easily separated from reactants and products by depressurization, leaving virtually no residues on the reaction products. Oliveira (*13*) and Marty (*14*) compared lipase transesterification reactions in hexane and in $scCO_2$ and reported similar conversions and reaction rates in both media. Other researchers observed an enhancement of activity in $scCO_2$ (*15*) and/or selectivity near the critical point (*16*). The main disadvantage of using $scCO_2$ as reaction solvent for oil systems is that relatively large amounts of carbon dioxide at several hundred bars are required to dissolve all reactants in the gas phase. An approach to overcome this drawback is dissolving carbon dioxide in the reaction mixture, rather than dissolving the reactants in carbon dioxide. A gas-expanded liquid medium (GXL), which has lower viscosity than the original reaction medium (*17*), is formed at relatively low pressures. For a lipase-catalyzed esterification reaction, Laudani et al. (*18*) showed that the reaction rate in GXL depended on pressure and that it was higher than in solvent-free and hexane media. Yet, there are not many studies on the effect of GXL on activity and especially selectivity of lipases.

In this work, we studied milk fat ethanolysis in different reaction conditions with the objective of maximizing the transesterification of short-chain fatty acids from milk fat to the FAEE product, to form a mixture enriched in short-chain flavor esters. Three commercial lipases, Novozym 435, Lipozyme TL, and Lipozyme RM, featuring different selectivity types (positional and chain-length selectivity), were to this end tested and compared in solvent-free hexane and the particularly interesting carbon dioxide-expanded liquid medium.

MATERIALS AND METHODS

Materials. Concentrated milk fat (98% milk fat, olein fraction) was stored at 4 °C before use. Absolute ethanol (99.9%), tributyrin (>97%), and sodium ethoxide solution (21 wt % in ethanol) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Ethyl butyrate (99.5%), hexanoate (99.8%), octanoate (99.8%), decanoate (99.5%), myristate (98.5%), laurate (99.8%), palmitate (99.8%), oleate (99.5%), and stearate (99.5%), used as analytical standards, were also purchased from Sigma-Aldrich. Carbon dioxide was from Linde Gas Benelux (Schiedam, The Netherlands).

The enzymes Lipozyme TL IM, Lipozyme RM IM, and Novozym 435 were donated by Novozymes A/S (Bagsvaerd, Denmark). Novozym 435 is *Candida antarctica* lipase type B, immobilized on macroporous acrylic resin beads (300−900 μm). Lipozyme RM IM is *Rhizomucor miehei* lipase, immobilized on anion exchange resin particles (~600 μm). Lipozyme TL IM is *Thermomyces lanuginosa* lipase immobilized on granulated silica particles (300−1000 μm). IMMCALB-T1-050 (*C. antarctica* lipase B immobilized by adsorption on granulated silica with particle size ca. 50 μm) was from Chiral Vision (Leiden, The Netherlands).

The tributyrin hydrolysis activity of all lipases used was measured as follows: Equimolar mixtures of tributyrin and water were vigorously shaken and incubated at 45 °C with 2 wt % of lipase. The residual water present in the mixture was measured at regular time intervals, using Karl Fischer titration. The initial hydrolytic activity measured is expressed as μmol of butyric acid released per min (U) and per g of enzyme: 2400 U/g for Novozym 435, 3350 U/g for Lipozyme RM, 3150 U/g for Lipozyme TL, and 2600 U/g for IMMCALB.

Determination of Milk Fat Fatty Acid Composition. The fatty acid composition of the milk fat fraction used in the experiments was determined in two independent assays by which known amounts of milk fat were completely transesterified to FAEE with excess ethanol and sodium ethoxide (diluted to 2.5 wt %) as the catalyst. The reactions were run for 24 h at 45 °C. Aliquots of the product mixtures were analyzed in triplicate by gas chromatography. The resulting average fatty acid molar composition of the milk fat fraction used is given in **Table 2**. Only the nine major milk fat fatty acids (representing 90−95% of the total milk fat fatty acids) were quantified, so the measured composition of milk fat was normalized to those nine fatty acids. The molecular weight of the milk fat used was estimated to be approximately 770 g/mol.

Table 2. Fatty Acid Composition of the Milk Fat Fraction Used in the Experiments

fatty acid	mol %
butyric (C4)	10.1 ± 0.4
caproic (C6)	5.2 ± 0.3
caprylic (C8)	2.6 ± 0.2
capric (C10)	4.8 ± 0.3
lauric (C12)	5.1 ± 0.1
myristic (C14)	11.7 ± 0.1
palmitic (C16)	27.4 ± 0.9
stearic (C18)	10.2 ± 0.4
oleic (C18:1)	22.9 ± 0.4

Solvent-Free and Hexane Transesterifications. Transesterifications without solvent and in hexane were performed in 20 mL minireactors provided with a magnetic stirrer and kept at 42−45 °C in a thermostatic oven. Ten grams of milk fat and variable amounts of ethanol were used. The molar ratio of reactants varied between 0.4 and 2 (ethanol to total fatty acids). The concentration of enzyme was 2 wt % in relation to the fat used. For the experiments in hexane, approximately 10 g of hexane was added to dissolve the reactants mixture. For monitoring the reactions, samples of ca. 10 mg were regularly withdrawn from the minireactors and dissolved in 1.5 mL of hexane for GC analysis.

GXL Transesterifications. A jacketed stirred autoclave (Autoclave Engineers) was used for the transesterification in GXL medium. The autoclave had a volume of 385 mL and was equipped with two sapphire windows, a bottom mesh filter, and several inlet/outlet ports. In regular experiments, a certain amount of melted fat, enzyme, and ethanol was introduced in the autoclave, and then, it was closed and pressurized until only one phase (expanded liquid phase) was observed through the sapphire windows. An estimation of the necessary amount of reactants was made beforehand so that the mixture of reactants and carbon dioxide became homogeneous at 100−110 bar. The temperature was kept at 42 °C, and stirring was performed by a six-blade turbinelike impeller at 150 rpm. Samples from the reaction mixture, free of lipase particles, were taken regularly by opening the bottom valve.

Analysis. Analyses were conducted on a gas chromatograph Chrompack CP9002 (Varian Inc., Middelburg), equipped with flame ionization detector (FID), using a VF5-ht GC column (Varian Inc.). Injection and detection temperatured were 350 °C. N_2 was the carrier gas. The oven temperature was kept at 50 °C for 2 min, then raised at 20 °C/min to 350 °C, and kept for 2 min at the final temperature. This temperature profile allowed elution of all compounds and quantification of FAEE.

RESULTS AND DISCUSSION

Enzyme Type. FAEE production was monitored over time for milk fat ethanolysis reactions catalyzed by each enzyme type (Lipozyme TL, Lipozyme RM, and Novozym 435). Product formation (F) is expressed as moles of FAEE produced over total moles of fatty acids (FA) in the original milk fat. The percentage of short-chain FAEE in the whole FAEE product mixture is expressed as the short-chain relative ratio, r_{SC}, defined as the mol fraction of C4−C12 fatty acids in the actual FAEE product over the mol fraction of C4−C12 fatty acids in the original milk fat, as shown in the following equation:

$$r_{SC} = \frac{x_{C4-C12,FAEE}}{x_{C4-C12,TG(0)}}$$

Thus, short-chain selectivity results in $r_{SC} > 1$, while long-chain selectivity results in $r_{SC} < 1$.

Figure 1 shows the reaction progress and the short-chain relative ratio in an ethanolysis experiment catalyzed by 2 wt % of Novozyme 435 (NV). All data points were obtained as the average of three measurements. The maximum product formation was limited by the amount of ethanol, as the ethanol/fatty acid ratio (E/FA) was 0.9. Lipase selectivity for short-chain fatty acids was observed especially during the first hours of reaction, where r_{SC} reached a value of 1.4. The residual partial glycerides were therefore enriched in long-chain fatty acids. Later, as the reaction proceeded, r_{SC} decreased steadily to values around 1.1. NV lipase is reported to be nonregiospecific and, because of the limited space available of its active site, to show certain selectivity for short-chain fatty acids (*5*, *19*). However, this short-chain selectivity is not absolute, as this would result in a FAEE product composed solely of short chains. Instead, it appears that NV releases short-chain fatty acids (small substrates) faster than longer chains.

Figure 2 compares the FAEE product composition obtained with each enzyme type after 2 h and after 25 h of reaction. For NV, enrichment in short chains in the FAEE product is observed during the first hours of reaction. On the contrary, the proportion of short-chain fatty acids in the FAEE mixture produced by both Lipozymes (TL and RM) was very low during the first hours of reaction and increased with conversion. This means that TL and RM transesterified long-chain fatty acids faster than short-chain fatty acids. Lipozyme RM and Lipozyme TL are known to be *sn*-1,3 specific (*5*, *20*), although, in general, the selectivity of enzymes may vary considerably depending on the

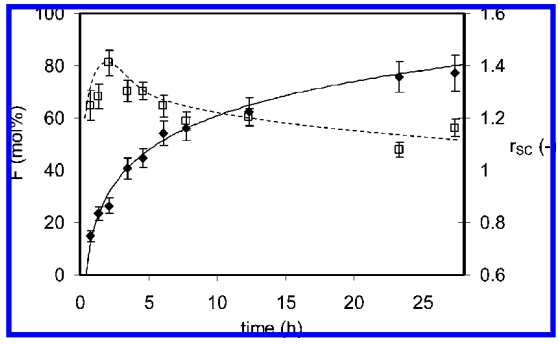

Figure 1. Product formation *F* (black rhombus) and relative short-chain ratio r_{SC} (empty squares) vs time for an ethanolysis reaction catalyzed by NV. The enzyme load was 2 wt %, and E/FA was 0.9.

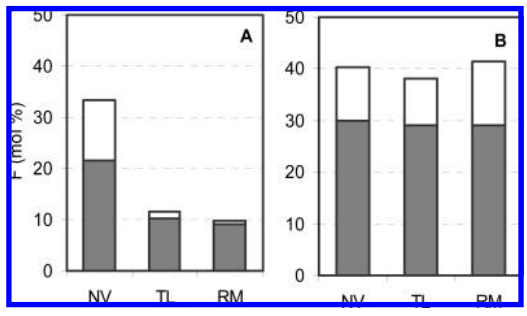

Figure 2. Product formation *F* and composition of the FAEE mixture produced by each of the lipases tested, after 2 (**a**) and 25 h (**b**) of reaction. White bars, short-chain fatty acids (C4−C12); dark gray bars, long-chain fatty acids (C14−C18). The enzyme load was 2 wt %, and E/FA was 0.4.

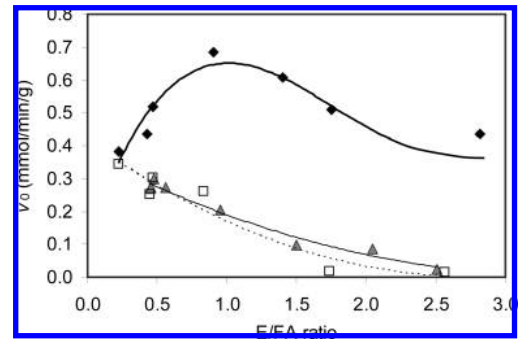

Figure 3. Initial enzymatic activity per enzyme unit, v_0 (mmol/min/g) vs reactants ratio, E/FA. Black rhombus, Novozym 435; empty squares, Lipozyme RM; and gray triangles, Lipozyme TL. The enzyme load was 2 wt % in all experiments.

reaction system and immobilization type (*21*). The milk fat fraction used in our experiments contained approximately 28 mol % short- (and medium-) chain fatty acids, C4−C12, mostly esterified in the *sn*-1,3 positions. Therefore, the FAEE mixture produced using a strict *sn*-1,3 lipase should contain roughly 40 mol % short-chain FAEE. However, this was not the case: The enrichment in long-chain fatty acids observed in **Figure 2a** means that RM and TL converted *sn*-1,3 long chains faster than *sn*-1,3 short chains. Possibly, these lipases show long-chain selectivity within *sn*-1,3 selectivity, resulting in a long-chain fatty acid enrichment in the initial FAEE product.

On the other hand, the final conversion obtained with the three lipases was practically identical, which is logical because the presence of the enzyme does not affect the equilibrium conversion. The final r_{SC} values reached by all three lipases were close to 1, namely, between 0.9 and 1.1, resulting in a FAEE composition practically identical to that of the original milk fat. This effect is likely due to randomization of the FAEE product over time. All reaction steps in the ethanolysis of TGs are reversible, according to the following scheme:

$$TG + E \leftrightarrow DG + FAEE$$
$$DG + E \leftrightarrow MG + FAEE$$
$$MG + E \leftrightarrow G + FAEE$$

The reaction product at every moment of time consists of a mixture of FAEE, monoglycerides (MG), diglycerides (DG), free glycerol (G), and unconverted TG. FAEE may also react back until the rate of FAEE formation is identical to the rate of FAEE consumption. In addition, it is known that acyl migration reactions occur spontaneously, transforming *sn*-2 into *sn*-1(3) MG and *sn*-1,2 DG into *sn*-1(3),2 DG (*22*). All of these phenomena result in the randomization of FAEE product, and eventually r_{SC} in the FAEE product may become equal to an analogous r_{SC} in the partial glycerides residue.

The initial activity of TL and RM was in general lower than that of NV in ethanolysis reactions, as shown in **Figure 3**, although the opposite was observed during the tributyrin hydrolysis tests (Materials and Methods). A possible explanation for the low activity of TL and RM in ethanolysis reactions is ethanol inhibition, favored by the nature of their carrier material. The presence of alcohols generally inhibits lipase activity in esterification and transesterification reactions (*8*) because it reversibly binds to the active site of the lipase. TL and RM lipases are immobilized on hydrophilic carriers, to which polar ethanol attaches more easily than the more hydrophobic glycerides do. They may therefore be more susceptible to suffering ethanol inhibition. In addition, the probability that the lipase active site meets acyl donors (glycerides) becomes lower in this situation.

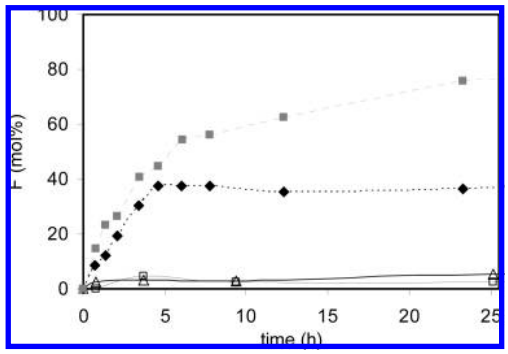

Figure 4. Product formation *F* vs time. Empty squares, IMMCALB lipase with E/FA = 0.4; empty triangles, IMMCALB lipase with E/FA = 0.9; black rhombus, NV lipase with E/FA = 0.4; and gray squares, NV lipase with E/FA = 0.9. The enzyme load was 4 wt % for IMMCALB experiments and 2 wt % for NV experiments.

Reactants Ratio. The effect of the reactants ratio on lipase activity and selectivity was investigated by performing ethanolysis experiments varying the concentration of ethanol, expressed as the ratio of ethanol to initially bonded fatty acids (E/FA). The initial activity, v_0 (mmol/min/g enzyme) of both Lipozyme RM and Lipozyme TL decreased with ethanol concentration, as shown in **Figure 3**, as a probable consequence of ethanol inhibition. For NV, the initial activity was maximum around the stoichiometric ratio (E/FA = 1) and decreased at higher E/FA. Although this is not necessarily evidence of ethanol inhibition (the conversion rate is maximal at the stoichiometric ratio for first-order kinetics with respect to each of the reactants), many authors have shown that the mechanism of enzyme-catalyzed acyl exchange reactions is successfully explained by the Ping Pong bi bi model with competitive alcohol inhibition (*8*). According to this model, the reaction rate v_i depends on the concentrations of both reactants [ethanol (E) and glyceride-bonded fatty acids (FA)] and on four parameters: v_{max} (maximum reaction rate), k_{mE} and k_{mFA} (apparent affinity constants of ethanol and glyceride-bonded fatty acids, respectively), and K_i (apparent inhibition constant of ethanol):

$$v_i = v_{max}\frac{[FA]\cdot[E]}{[FA]\cdot[E] + k_{mE}[FA] + k_{mFA}[E]\cdot\left(1+\frac{[E]}{K_i}\right)}$$

This type of kinetic equation can explain satisfactorily the effects of reactants ratio on the initial activity shown in **Figure 3**. Assuming Ping Pong bi bi kinetics for the three enzymes, it becomes clear that ethanol inhibited NV to a much lower extent than the other two lipases; that is, for NV, the apparent inhibition constant K_i was larger. The hydrophobic nature of the carrier material of NV would explain the weaker dependence of its activity with ethanol concentration, as compared to the other two lipases. The apolar polyacrylic beads are likely to be surrounded by the most hydrophobic fatty compounds of the mixture, which partially protect the lipase active sites from contacting the inhibitory ethanol molecules.

This hypothesis was checked by testing the performance of *C. antarctica* lipase B immobilized on hydrophilic silica particles (IMMCALB) in the ethanolysis reaction. The results shown in **Figure 4** reveal that the activity of hydrophilic IMMCALB was dramatically lower than that of its hydrophobic counterpart NV, while both lipases showed similar activity in the tributyrin hydrolysis test (2600 and 2400 U/g, respectively). These observations confirm that the hydrophobic or hydrophilic nature of the lipase carriers has a very important effect on the lipase catalytic performance.

Table 3. Average r_{SC} up to 40% Conversion for Solvent-Free Ethanolysis Catalyzed by Novozym 435 at Different E/FA Ratios[a]

experiment	lipase	medium	E/FA	average r_{SC}
A-1	NV	solvent-free	0.4	1.31
A-2	NV	solvent-free	0.9	1.38
A-3	NV	solvent-free	1.4	1.45
A-4	NV	solvent-free	2.8	1.53

[a] The average r_{SC} values are calculated using a polynomial curve fitted to the experimental data (r_{SC} vs *F*). The standard deviation of all r_{SC} values is between 0.05 and 0.10.

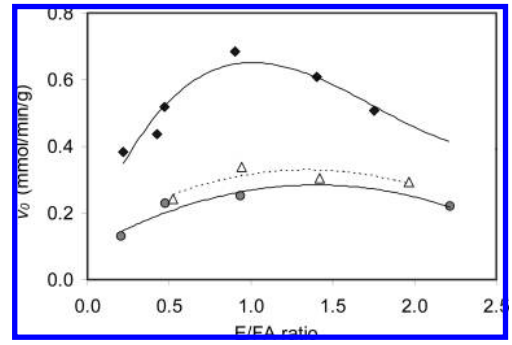

Figure 5. Initial activity of NV (v_0) vs ratio of reactants (E/FA) in different solvents. Black rhombus, solvent-free; gray circles, hexane; and white triangles, GXL at 110 bar. In all cases, the enzyme load was 2% wt. GXL experiments were performed in the high pressure setup, while solvent-free and hexane experiments were run in laboratory scale.

Table 3 shows the average value of r_{SC} up to 40% conversion for solvent-free ethanolysis reactions performed by NV at different E/FA ratios. In general, it appears that the selectivity of NV for short-chain fatty acids increased with ethanol concentration. This can be related to the fact that increasing concentrations of ethanol reduce the overall viscosity of the mixture, thus contributing to an enhancement of internal diffusion. This effect is discussed in more detail in the next section.

Solvent Medium. Transesterification reactions catalyzed by Novozym 435 in (1) hexane and (2) under dissolved carbon dioxide at a pressure of 110 bar (GXL) were compared to analogous solvent-free experiments. As shown in **Figure 5**, the use of solvents resulted in a decrease of the product formation rate in relation to the solvent-free experiments. This is a probable consequence of the decrease of reactants concentration by dilution. In addition, it can be observed that the initial product formation rate depended strongly on the concentration of ethanol in the solvent-free experiments but not on the presence of hexane or dissolved carbon dioxide. Therefore, it appears that the concentration of ethanol does not have an important effect on the reaction rate when the reactants are diluted by a solvent (hexane) or cosolvent (carbon dioxide). The availability of acyl donors (glycerides) surrounding the enzyme particles seems instead to be the main factor determining the reaction rate. On the other hand, the final conversion was practically the same in all media for a given ratio of reactants, meaning that the presence of a solvent did not affect the reaction equilibrium (results not shown).

It is important to stress that not all of the data points presented in **Figure 5** were produced in the same experimental setup. GXL experiments were performed in the high pressure setup, while solvent-free and hexane experiments were run in magnetically stirred glassware reactors. Because of the geometry of the high pressure reactor, a fraction of the enzyme load was deposited on a dead volume; therefore, the overall reaction rate was

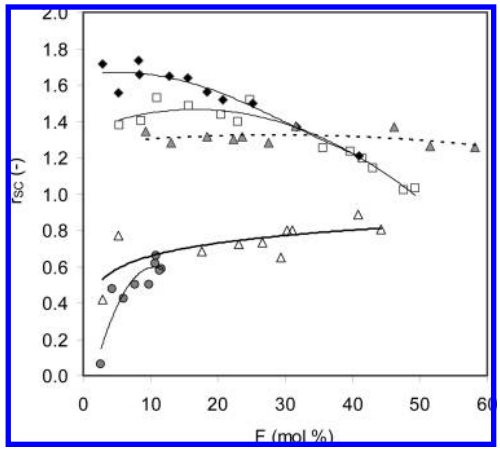

Figure 6. r_{SC} vs product formation *F*. Gray circles, RM in GXL with E/FA = 0.5; white triangles, TL in GXL with E/FA = 0.5; black rhombus, NV in GXL with E/FA = 0.5; empty squares, NV solvent-free with E/FA = 0.5; and gray triangles, NV in GXL with E/FA = 1.4. All experiments were performed in the high pressure setup using 2 wt % enzyme load.

Table 4. Average r_{SC} up to 40% Conversion for Solvent-Free Ethanolysis Catalyzed by Novozym 435 in Different Solvent Media[a]

experiment	lipase	medium	E/FA	average r_{SC}
CO-A-0	NV	solvent-free	0.5	1.36
H-1	NV	hexane	0.5	1.47
H-2	NV	hexane	0.9	1.64
CO-A-1	NV	GXL	0.5	1.53
CO-A-3	NV	GXL	1.4	1.31

[a] The average r_{SC} values are calculated using a polynomial curve fitted to the experimental data (r_{SC} vs *F*). The standard deviation of all r_{SC} values is between 0.05 and 0.10.

reduced to some extent. As a consequence, it is not possible to compare the absolute initial activities measured in the high pressure vessel to those measured in the laboratory reactors. Two facts are, however, clear: The influence of ethanol concentration on activity is stronger in solvent-free medium than in diluted media, and the maximum activity of NV lipase is found in all cases around E/FA = 1.

Ethanolysis experiments in GXL at a pressure of 110 bar were also run with TL and RM lipases using ethanol to fat ratio E/FA = 0.5. **Figure 6** shows r_{SC} vs product formation in GXL experiments using all three lipases. A considerable reduction in the activity of Lipozyme RM was observed in GXL. The FAEE product formation was only 10−12% after 9 h, and this resulted in a low final r_{SC} value (approximately 0.6), because for this enzyme, r_{SC} increases with conversion. A possible reason for the decrease of RM activity in GXL is that it suffered deactivation by the high pressure applied, phenomenon also reported for this lipase by other researchers (*23*). On the other hand, and as already observed in solvent-free experiments, r_{SC} in GXL ethanolysis experiments catalyzed by NV was remarkably higher than that of the other two enzymes, at all conversion levels.

The effect of solvents on Novozym 435 selectivity is shown in both **Figure 6** (GXL experiments) and in **Table 4** (GXL and hexane experiments), which also include the results of a solvent-free ethanolysis experiment catalyzed by NV in the high pressure setup (CO-A-0). **Table 4** compiles the average r_{SC} values up to 40% conversion for ethanolysis in different media. The relative short-chain ratio (r_{SC}) in the FAEE mixture produced by NV was in general higher in diluted media than in solvent-free conditions. For experiments at E/FA = 0.5, average r_{SC} reached 1.47 in hexane and 1.53 in GXL, while the solvent-free average value was 1.36. A possible explanation for this effect is based on the medium viscosity and the different accessibility of external active sites (located on the enzyme surface) and internal ones (located inside the pores of the carrier). The following expression proposed by Geankopolis gives the (external) diffusion coefficient for relatively large biological solutes in liquid solvents (*24*). The diffusion coefficient increases with temperature (*T*) or by decreasing the medium viscosity (μ_B) and decreases with increasing molecular weight of the solute (M_A).

$$D_{AB} = \frac{9.5 \times 10^{-5} \cdot T}{\mu_B \cdot M_A^{1/3}}$$

By diluting the reaction medium, the overall medium viscosity decreases; thus, an enhancement of diffusivity of both small TGs (containing short-chain FA) and large TGs (containing long-chain FA) results. On the other hand, the effective diffusion coefficient inside the pores depends on the enzyme particles porosity (ϵ) and pore tortuosity (τ) and on a restrictive factor, K_r, which accounts for the effect of the pore diameter (d_p), causing interfering collisions of the diffusing solutes with the pore wall (*24*):

$$D_e = \frac{D_{AB} \cdot \varepsilon}{\tau} \cdot K_r$$

$$K_r = \left(1 - \frac{d_m}{d_p}\right) \quad \frac{d_m}{d_p} \leq 1$$

As K_r comes close to zero, when the solute diameter d_m approaches the pore diameter d_p, there might be a size exclusion effect on the larger TGs, which cannot reach the active sites into sufficiently small pores. As a consequence, internal diffusion of only the smaller acyl donors would be enhanced by decreasing the medium viscosity, resulting in an apparent enhancement of the enzyme selectivity, as reflected by the higher average r_{SC}. However, if we compare the volume of the largest TG likely to be present in the mixture (triolein, 1.6 nm^3) (*25*) to the volume of the crude protein CAL-B (between 4.0 and 8.5 nm^3) (*26*), it can be concluded that size exclusion effects are not likely to occur. As CAL-B is much larger than triolein, it does not seem possible that the lipase can be present in pores small enough not to be accessible for large TGs.

Another explanation for the enhanced short-chain selectivity in the diluted medium can be given assuming that the reaction on short chains is inherently faster than the reaction on long chains. This would be reflected in different values of v_{max} and k_{mFA} for substrates containing short or long fatty acids. This phenomenon is encountered and even applied, for example, on the enzymatic kinetic resolution of enantiomers (*27*). If the reaction is diffusion-limited, the rate of the fast-reacting substrate is affected in relatively larger extent (*27*), as the apparent reaction rates of all substrate types are leveled off by mass transfer limitation. When diffusion is facilitated as, in this case, by adding a low-viscosity solvent, the faster reacting substrates (short-chain glycerides) benefit the most by the shift from a diffusion-controlled to a conversion-controlled reaction. In addition, the reaction of long-chain TGs may remain diffusion-controlled to some extent, since its corresponding diffusion coefficient D_{AB} is smaller. These effects can explain the increase of the relative short-chain ratio r_{SC} in diluted media observed in our experiments.

The increase of r_{SC} in hexane was even more remarkable at high ethanol to fatty acid ratios (E/FA = 0.9), possibly because the reaction rate becomes close to its maximum in these

conditions. Again, this favors in larger extent the less diffusion-limited substrates (short chains). On the other hand, as shown in **Figure 6**, the GXL ethanolysis catalyzed at higher ethanol concentration (E/FA = 1.4) resulted in lower and rather constant values of r_{SC} at all conversion levels (1.31 in average, **Table 4**). A possible reason for this difference is the fact that, in the presence of CO_2, the conformation of the lipase active site may be altered, due to covalent binding of CO_2 with lysine residues from the active site (*28*). This would result in a different behavior of the lipase in terms of selectivity, as compared to its performance in other solvent media (*28*). The CO_2 alteration effect seems to take place only at high ethanol concentrations, possibly due to additional CO_2−ethanol interactions.

Among all of the experimental conditions tested, the highest r_{SC} values were obtained using NV lipase in hexane and in GXL at low E/FA ratios. The use of CO_2 as cosolvent (GXL) allows reaching high r_{SC} in partial ethanolysis without the need of using excess ethanol. The fact that the use of GXL is able to enhance the short-chain selectivity of NV is interesting from a processing point of view. On one hand, the only solvent used is CO_2, which can be easily separated from the products mixture by depressurization. Besides, CO_2 is nontoxic, and it does not leave any residue on the product. This is especially important when considering food applications. On the other hand, selective removal of products (short-chain FAEE) from the reaction mixture can be carried out using CO_2 as extraction solvent. Because the reaction selectivity is at the same time enhanced using CO_2 as cosolvent, the possibility of integrating reaction and separation appears as an attractive processing option.

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