Enzymatic Production of Alkyl Esters Through Alcoholysis: A Critical Evaluation of Lipases and Alcohols

Li Deng[a,b], Xuebing Xu[a,*], Gudmundur G. Haraldsson[c], Tianwei Tan[b], and Fang Wang[b]

[a]BioCentrum-DTU, Technical University of Denmark, 2800 Kgs., Lyngby, Denmark, [b]Department of Biochemical Engineering, Beijing University of Chemical Technology, Beijing, China, and [c]Science Institute, University of Iceland, Dunhaga 3, 107 Reykjavik, Iceland

ABSTRACT: This paper focuses on a detailed evaluation of commercially available immobilized lipases and simple monohydric alcohols for the production of alkyl esters from sunflower oil by enzymatic alcoholysis. Six lipases were tested with seven alcohols, including straight and branched-chain primary and secondary alcohols. The reactions were conducted in a batch stirred reaction vessel using stoichiometric amounts of substrates under solvent-free conditions. Dramatic differences in alcoholysis performance were observed among the different lipases. For most of the alcohols, Novozym 435 produced the highest yield of FA alkyl esters, with yields well over 90% for methanol, absolute ethanol, and 1-propanol. Overall, 96% ethanol was the preferred alcohol for all lipases except Novozym 435, and ethanolysis reactions reached the maximal conversion efficiency. Increasing the water content in the system resulted in an increased degree of conversion for all lipases except Novozym 435. The secondary alcohol 2-propanol significantly reduced the alcoholysis reaction with all lipases; however, the branch-chain isobutanol was more advantageous than linear 1-butanol for Novozym 435, Lipozyme RM IM, and Lipase PS-C. Many commercial immobilized lipases are highly efficient and promising for the production of alkyl esters, offering high reaction yields and a simple operation process.

Paper no. J11001 in *JAOCS 82*, 341–347 (May 2005).

KEY WORDS: Alcohols, alcoholysis, alkyl esters, batch reaction, biodiesels, lipases.

FA alkyl esters play important roles as technical chemicals and food ingredients. Esters of long-chain FA are of increasing economic interest in many industries and involve a wide range of applications (1). FA monoesters of short-chain alcohols (C_1–C_4), such as isopropyl palmitate, are used extensively in cosmetic and topical medicinal preparations because of their good biocompatibility and absorption through the human skin (2). They also can be used as diesel fuel substitutes, offering physical and chemical properties similar to fossil diesel fuel. FAME production has been studied extensively because of the increasing attractiveness of the resulting environmentally friendly biodiesels (3). Recent government mandates in the United States and Brazil have added impetus to the development of cost-effective routes for the synthesis of biodiesels. Alkyl monoesters of FA are also used as the preferred form for the fractionation of FA, for example, in the fish oil industry, either through urea complexation or short-path distillation (4).

The commercial production of the various products mentioned is based mostly on chemical methods. Lipases (TAG acylhydrolases E.C. 3.1.1.3) have been used as biocatalysts as an alternative route to the conventional chemical processes (2). Biocatalysis offers numerous merits over the chemical processes, such as mild reaction conditions, high specificity, and so on. The lipases have been widely accepted as biocatalysts for the modification of oils and fats (5–7). Recently, the lipase-catalyzed alcoholysis of vegetable oils and animal fats for the production of FA methyl or ethyl ester biodiesels has been reported (8–13). However, these studies have been conducted under individual conditions and offered limited information on the reaction activity and performance of different lipases with various alcohols. This often limits the exploitation of these ideas for different process setups and different product developments based on enzymatic methods.

In this paper, six immobilized lipases from commercial sources were used for the alcoholysis of sunflower oil. Alcohols such as methanol, ethanol, 1- and 2-propanol, 1-butanol, and isobutanol were selected for a systematic comparison. The reactions were conducted batchwise under solvent-free conditions using only stoichiometric amounts of the substrates. The behavior and performance of different lipases with different alcohols were investigated and compared in the survey.

MATERIALS AND METHODS

Materials. Lipozyme TL IM (*Thermomyces lanuginosa* lipase, TLL-1), Lipozyme RM IM (*Rhizomucor miehei* lipase, RML), and Novozym 435 (*Candida antarctica* lipase B, CAL) were donated by Novozyme A/S (Bagsvaerd, Denmark). Lipase LA201 was supplied by Novozyme A/S for comparison reasons and is a *T. lanuginosa* lipase (TLL-2) immobilized on a polymer carrier (polypropylene) with a 3.50 wt% water content. (Water content in the lipases and alcohols as well as in the oil in this description was determined by Karl

*To whom correspondence should be addressed at BioCentrum-DTU, Technical University of Denmark, Building 221, 2800 Kgs., Lyngby, Denmark. E-mail: xx@biocentrum.dtu.dk

Fischer titration.) Lipozyme TL IM and Lipozyme RM IM are commercial 1,3-regiospecific lipases. The former lipase is immobilized on granulated silica and the latter on a macroporous ion-exchange resin. The water content of the two lipases is 5.82 and 3.04 wt%, respectively. Novozym 435 is a nonregiospecific lipase immobilized on a macroporous acrylic resin with a water content of 1.92 wt%. Lipase PS-C (*Pseudomonas cepacia* lipase, PCL) and Lipase AK-C (*Pseudomonas fluorescens* lipase, PFL) were donated by Amano (Nagoya, Japan). The former is a commercial nonregiospecific lipase immobilized on ceramic particles with a 4.03 wt% water content. Lipase AK-C is a 1,3-regiospecific lipase immobilized on ceramic particles with a 1.97 wt% water content.

Refined high oleic acid sunflower oil, with a water content of 0.072%, was provided by Karlshamns AB (Karlshamn, Sweden). Methanol (99.8%, water content 0.001%), ethanol (99.9%, water content 0.001%), 96% ethanol (water content 4.013%), 1-propanol (99.5%, water content 0.048%), 2-propanol (99.7%, water content 0.105%), 1-butanol (99.5%, water content 0.097%), and hexane were purchased from Merck KGaA (Darmstadt, Germany). Isobutanol (GC 99.9%, water content 0.098%) was from Buck & Holm (Herlev, Denmark). Ethyl acetate and acetic acid (99%) were from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Cupric sulfate ($CuSO_4 \cdot 5H_2O$) and phosphoric acid (85%) were purchased from Sigma Chemical (St. Louis, MO). The high-performance TLC (HPTLC) plates (20 × 10 cm; Silica gel 60) were purchased from Merck KGaA and were activated in an oven before use. Monoolein (99%, 1-monooleoyl-*rac*-glycerol), diolein (85% 1,3- and 15% 1,2-DAG), triolein (99%), and ethyl oleate (99%) were from Sigma Chemical and were used as standards in the HPTLC analysis. All chemicals and reagents for the analysis were of analytical or chromatographic grade.

Alcoholysis of sunflower oil. The reactions were run with 20 g of oil in a water bath equipped with a magnetic stirrer. Alcohols were added to the oil according to the designed amount and adding procedure. Immobilized lipase (2 g) was added to the mixture to start the reaction. The substrate ratio was kept at a stoichiometric amount (3 molar equivalents alcohol to 1 mol TAG). The alcohols were added stepwise to the reaction evenly in four lots (1/4 each time) to minimize the inhibition of alcohol, as studied previously (8,13): the first portion at the beginning of the reaction, the second portion after 2 h, the third portion after 4 h, and the fourth portion after 8 h. The reaction was allowed to proceed for an additional 16 h, providing an overall reaction time of 24 h. The reaction temperature was maintained at 40°C. Samples (1 mL) were withdrawn after 2, 4, and 8 h, just before the addition of alcohols, and the final sample was withdrawn after 24 h.

Analysis by HPTLC. The progress of alcoholysis was monitored by an HPTLC method with modifications for the developing solvent system (14). The HPTLC system (Desaga GmbH, Wiesloch, Germany) consisted of an AS 30 TLC applicator, a Densitometer CD 60 scanner, and ProQuant Control and Evaluation software. Samples (7 μL) from the reaction mixture were dissolved in hexane (1 mL). The AS 30 applicator was used to introduce the samples to the plate. The plate was developed with a developing solvent consisting of hexane/ethyl acetate/acetic acid (90:10:1, by vol). After development, the plate was thoroughly dried and sprayed with an aqueous solution of phosphoric acid (9.4 vol%) and cupric sulfate (15.6 wt%). After drying, the CD 60 scanner was used to quantify the bands. Each band was identified by comparison with standards. The weight of each band was calibrated and calculated with the oleic acid standards (ethyl oleate, monoolein, diolein, triolein, and oleic acid) from the peak area. The molar amount (M_i) was therefore calculated as based on the M.W. of the component. Duplicate measurements were made and the average was taken for all the analysis results, with a SD of less than 3.6%. With respect to forming FA alkyl esters, the yield of each lipid class, in molar percentage, can be better described as moles of acyl equivalents. The following equation was thus used to calculate the yield of each species:

$$\text{Yield}_i\ (\text{mol}\%) = \frac{n \times M_i}{3 \times M_{\text{TAG}} + M_{\text{FAAE}} + M_{\text{FFA}} + 2 \times M_{\text{DAG}} + M_{\text{MAG}}} \times 100\% \quad [1]$$

where FAAE is FA alkyl esters; *i* represents each component; and *n* is 1 for FAAE, FFA, and MAG, 2 for DAG, and 3 for TAG.

RESULTS AND DISCUSSION

The majority of the alcoholysis applications cited in the introduction involve ethanolysis, with ethanol as the acyl acceptor, specifically aiming at food applications. Obviously, ethanol is more readily accepted than other alcohols for environmental and toxicological reasons. Biotransformations involving lipases and alcohols warrant a few considerations, such as alcohol inhibition, water partition, and phase problems when oils and fats are involved. The most important concern for developing alcoholysis is to reveal how lipases perform in a simple operation system. Under a simple batch mixing system, the alcohol tolerance of lipases will be due to both inhibition by the alcohol and water partitioning.

In the present study, the lipase-catalyzed alcoholysis of sunflower oil was studied by investigating a range of simple monohydric alcohols including methanol, ethanol, 1-propanol, isopropanol, 1-butanol, and isobutanol. Furthermore, the influence of water was investigated in the case of ethanol by comparing absolute ethanol and 96% ethanol comprising 4% water. HPTLC has been widely used for lipid profile analyses (14). Compared with normal TLC, HPTLC gives much better and clearer separations. Vast amounts of useful information can be collected by that method with respect to analyzing a great number of samples in an efficient and reliable manner as the reactions proceed. The quantitative determination of alkyl esters produced; the individual acylglycerols MAG, DAG and TAG; and the FFA, indicative of hydrolysis side reactions, is possible (Table 1, Figs. 1–4).

TABLE 1
Evaluation of Lipases and Alcohols for the Production of Alkyl Esters[a]

				Glyceride profile at 24 h (mol%)				
Lipase	Alcohol	Type	Water[b]	FAAE	TAG	DAG	MAG	FFA
TLL-1	Methanol		6.43	89.8	0.6	2.5	4.3	2.8
	Ethanol	99%[c]	6.41	77.5	2.0	9.7	9.2	1.5
		96%	11.81	85.1	2.9	3.2	4.9	4.0
	Propanol	1-	6.47	78.1	2.2	7.1	10.1	2.4
		2-	6.55	24.6	29.3	26.8	14.3	5.0
	Butanol	1-	6.56	77.9	1.9	8.7	10.3	1.2
		Iso-	6.56	72.4	5.2	9.7	11.0	1.6
TLL-2	Methanol		4.13	68.4	4.1	13.8	12.0	1.6
	Ethanol	99%	4.11	66.9	2.9	13.4	14.7	2.2
		96%	9.51	70.7	6.5	10.4	8.6	3.8
	Propanol	1-	4.17	72.8	3.5	12.1	10.9	0.8
		2-	4.25	38.3	20.8	23.1	12.8	5.0
	Butanol	1-	4.26	64.2	1.3	13.6	19.0	2.0
		Iso-	4.26	59.9	6.9	15.6	14.6	3.0
RML	Methanol		3.63	59.1	14.1	12.8	12.5	1.5
	Ethanol	99%	3.61	70.2	11.4	8.6	9.1	0.7
		96%	9.01	79.1	4.5	5.7	6.9	3.8
	Propanol	1-	3.67	57.4	11.4	13.0	14.2	4.0
		2-	3.75	34.6	27.2	24.0	11.4	2.8
	Butanol	1-	3.76	60.4	13.7	9.8	15.1	1.0
		Iso-	3.76	73.0	6.6	9.7	9.2	1.6
CAL	Methanol		2.53	92.2	0.6	0.6	4.5	2.1
	Ethanol	99%	2.51	91.9	4.0	0.3	2.6	1.2
		96%	7.91	45.3	47.3	4.8	1.6	1.0
	Propanol	1-	2.57	93.2	1.3	1.9	2.3	1.3
		2-	2.65	77.8	3.8	7.6	8.8	2.0
	Butanol	1-	2.66	54.0	37.5	3.8	3.6	1.2
		Iso-	2.66	84.7	2.3	3.9	6.1	3.0
PCL	Methanol		4.63	28.2	36.0	20.7	12.1	2.9
	Ethanol	99%	4.61	29.9	28.3	26.6	14.6	0.7
		96%	10.01	88.4	1.2	3.9	3.6	2.9
	Butanol	1-	4.76	40.9	25.4	21.3	11.6	0.9
		Iso-	4.76	63.3	6.4	13.3	13.5	3.5
PFL	Methanol		2.63	8.4	61.4	21.7	5.1	3.4
	Ethanol	99%	2.61	30.0	29.2	22.7	15.8	2.3
		96%	8.01	45.3	10.7	20.7	17.9	5.3
	Butanol	1-	2.76	32.9	29.3	21.8	13.5	2.5
		Iso-	2.76	31.6	27.3	25.1	14.1	2.0

[a]Reaction conditions: 40°C, 10 wt% lipase dosage, addition of alcohol in four steps as described in the Materials and Methods section, 1:3 molar ratio of sunflower oil/alcohol.
[b]Water content in the reaction system was based on the lipase preparation (wt%). "Water" includes water in the lipase, oil, and alcohol.
[c]Ethanol types: 99%, indicating absolute anhydrous ethanol (0.001% water), and 96%, indicating 4% water. Abbreviations: TLL-1 and TLL-2, *Thermomyces lanuginosa* lipase (Lipozyme TL IM; Novozyme A/S, Bagsvaerd, Denmark); RML, *Rhizomucor miehei* lipase (Lipozyme RM IM; Novozyme A/S); CAL, *Candida antarctica* lipase (Novozym 435; Novozyme A/S); PCL, *Pseudomonas cepacia* lipase (Lipase PS-C; Amano, Nagoya, Japan); PFL, *Pseudomonas fluorescens* lipase (Lipase AK-C; Amano); FAEE, FA ethyl esters.

Methanolysis. Methanol is the most toxic and polar alcohol in the series and is commonly used for biodiesel production. Figure 1 shows the results of the methanolysis reaction for the lipases. CAL and TLL-1 offered the best performance and the highest conversion after 24 h (92 and 90%, respectively). Of all the alcohols tested, methanol was the best alcoholic substrate for TLL-1. TLL-2 and RML followed, with 68 and 59% conversions, respectively. The *Pseudomonas* lipases were the least active, with PCL and PFL offering only 28 and 8% conversions, respectively. Both lipases were virtually inactive after 2 h of reaction.

Ethanolysis. After 8 h, significant changes had taken place, and it became evident that both *Pseudomonas* lipases (PCL and PFL) were inferior under low water conditions (Fig. 2A). Their ethyl ester formation rate had diminished significantly after 4 h, at only 30% conversion, and remained around that level throughout the reaction. Three of the remaining lipases had reached conversion levels higher than 60%, with TLL-2 slightly behind at 50% conversion. After 24 h, it became clear that CAL was the most efficient lipase under these conditions, when 92% conversion into monoesters had been reached. The TLL-1, RML, and TLL-2 lipases remained well behind at 78, 70, and 67% conversion levels, respectively.

The poor performance of the *Pseudomonas* lipases possibly relates to the insufficient water content of the reaction medium

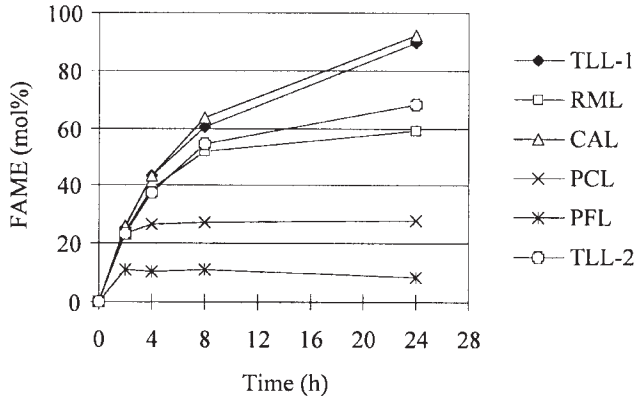

FIG. 1. Time courses of methyl ester formation catalyzed by different lipases in methanolysis. Reaction conditions: 40°C, 10 wt% lipase dosage, addition of methanol in four steps, sunflower oil (SFO) and methanol (1:3 mol/mol). Abbreviations: TLL-1 and TLL-2, *Thermomyces lanuginosa* lipase (Lipozyme TL IM; Novozyme A/S, Bagsvaerd, Denmark); RML, *Rhizomucor miehei* lipase (Lipozyme RM IM; Novozyme A/S); CAL, *Candida antarctica* lipase (Novozym 435; Novozyme A/S); PCL, *Pseudomonas cepacia* lipase (Lipase PS-C; Amano, Nagoya, Japan); PFL, *Pseudomonas fluorescens* lipase (Lipase AK-C; Amano).

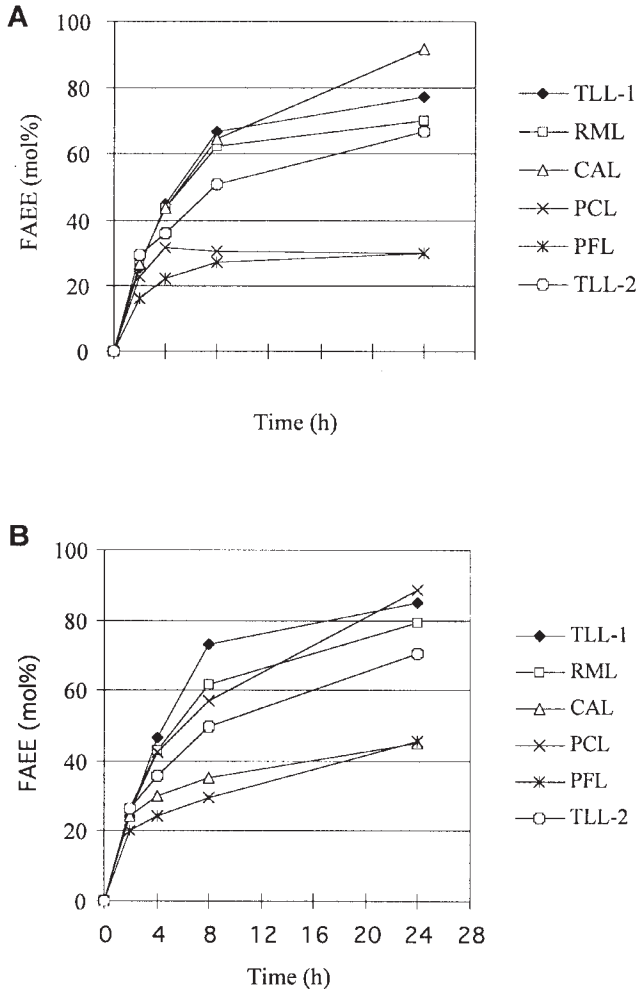

FIG. 2. Time courses of ethyl ester formation catalyzed by different lipases in ethanolysis. Reaction conditions: 40°C, 10 wt% lipase dosage, addition of ethanol in four steps. (A) SFO and anhydrous ethanol (1:3 mol/mol); (B) SFO and 96% ethanol (1:3 mol/mol). FAEE, FA ethyl esters; for other abbreviations see Figure 1.

for these lipases (Table 1). When enzymatic reactions are conducted in organic media, the water present in the reaction system plays a crucial role for maintaining optimal activity of the lipase (15,16). A certain amount of water is required for lipases to retain their optimal activity, but that amount appears to vary considerably among individual lipases, purely depending on the characteristics of each lipase. Under the present conditions, the highly polar ethanol tends to strip off the essential water from the lipase, resulting in diminished lipase activity in the case of PCL and PFL. The *C. antarctica* lipase, on the other hand, has been reported to tolerate similar reaction conditions (17) as well as to retain high activity under highly water-deficient conditions at high vacuum (18). Likewise, the silica-granulated *T. lanuginosa* lipase (TLL-1) is not affected by the water content in a single-phase fat–oil exchange system (19).

We anticipated that the increase of water in the system would help improve the reactivity of the enzymatic alcoholysis. Dramatic improvement in the performance of the *Pseudomonas* lipases took place when 96% ethanol (4% water content) was used (Fig. 2B). This was particularly evident for PCL, since that lipase offered the highest conversion level (88%) after 24 h (Table 1). An increase of water to the CAL (from 2.5 to 7.9%, based on the lipase; see Table 1) had the opposite effect. For that lipase, only 45% conversion into monoesters was obtained after 24 h. With both the TLL-1 and TLL-2 and the RML, slightly higher reactivities were obtained under higher water contents, as was previously observed with TLL-1 (17). RML has been shown to give better performance with a higher water content under similar reaction conditions (17). Our water level may have been below that needed for the increase.

In general, with 96% ethanol a higher conversion into ethyl esters was obtained after 24 h for all lipases except CAL (Fig. 2B). As illustrated in Figures 2A and 2B, the performance had nearly reversed in terms of monoester conversion for the low and high water content conditions. PCL displayed the highest activity and CAL displayed the lowest activity.

Hydrolysis is the side reaction associated with water content that, in general, leads to the formation of FFA. During the reaction, some of the initial water present in the system takes part in partial hydrolysis, and a compromise is often needed between optimal yields and the extent of hydrolysis side reactions. Such hydrolysis results in a lower water content and hence lower activity for some lipases. The essential water content available for lipases is also very much dependent on the reaction medium. Normally, the more polar the solvent, the more water is needed for optimal activity. On the other hand, more water will generally lead to the formation of higher FFA (Table 1). In this respect, a lipase with a lower water requirement is an advantage for the applications at issue in these studies.

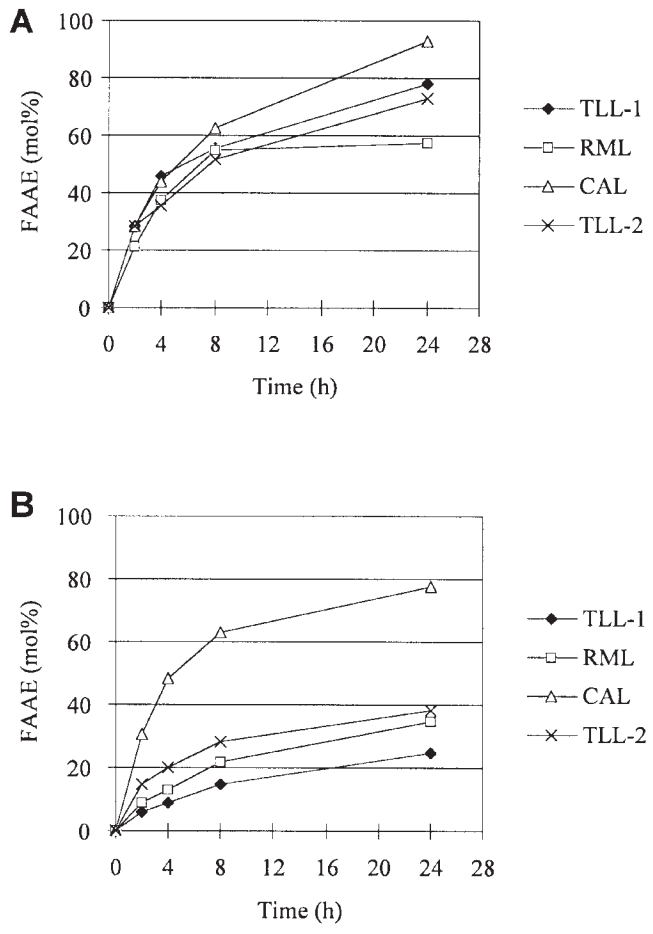

FIG. 3. Time courses of propyl ester formation catalyzed by different lipases in propanolysis. Reaction conditions: 40°C, 10 wt% lipase dosage, addition of propanol in four steps. (A) SFO and 1-propanol (1:3 mol/mol); (B) SFO and 2-propanol (1:3 mol/mol). For abbreviations see Figures 1 and 2.

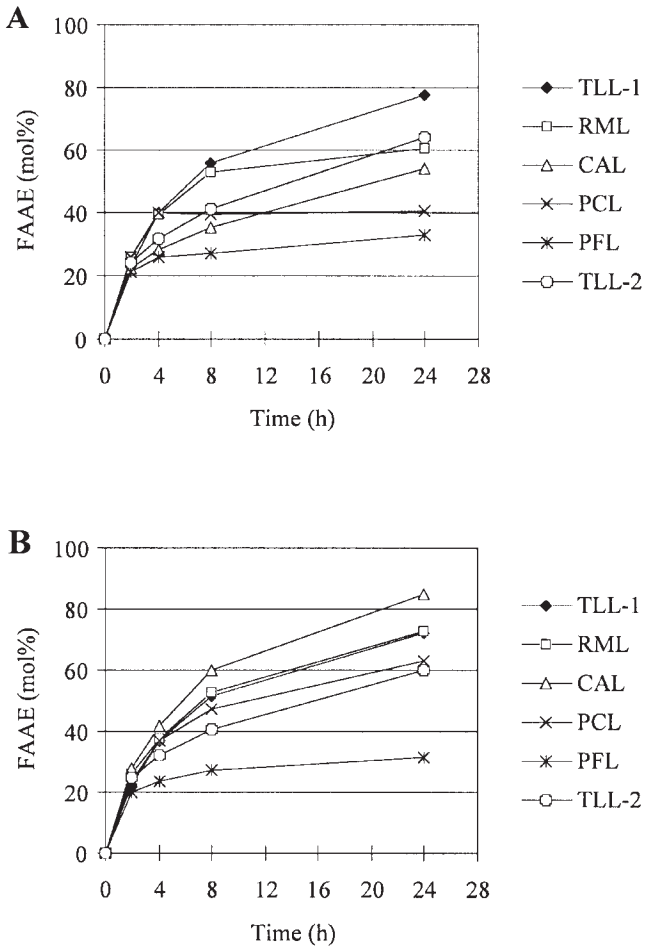

FIG. 4. Time courses of butyl ester formation catalyzed by different lipases in butanolysis. Reaction conditions: 40°C, 10 wt% lipase dosage, addition of butanol in four steps. (A) SFO and 1-butanol (1:3 mol/mol); (B) SFO and isobutanol (1:3 mol/mol). For abbreviations see Figures 1 and 2.

Propanolysis. Alcohols with alkyl chains longer than ethanol may be targeted for various reasons, such as better miscibility with oils and fats, less water partitioning and inhibition of lipases, and new application possibilities. With 1-propanol as the acyl acceptor, CAL presented the highest degree of monoester formation obtained in this study (93%), with TLL-1 and TLL-2 well behind (78 and 73%, respectively), as may be observed in Figure 3A. RML, on the other hand, did not significantly respond to the addition of the final quarter portion of 1-propanol and remained far behind, below the 60% conversion level.

With secondary 2-propanol as the acyl acceptor, all four lipases displayed far lower activity than with the primary 1-propanol (Fig. 3B). The conversion remained below 40% except for CAL, in which 75% monoesters were achieved after 24 h. That lipases usually prefer primary alcoholic substrates to secondary ones for steric reasons is well established. For CAL, the monoester formation rate in the first 8 h appeared to be slightly higher for 2-propanol than for 1-propanol. The high activity displayed by CAL on both propanols may be related to the claimed nonregiospecificity of that lipase toward lipase natural acylglycerol substrates and glycerol. However, it should be pointed out that under certain conditions, a lipase such as CAL, which usually is considered to be nonregiospecific, can display a high regiospecificity and act virtually exclusively at the 1,3-positions of the glycerol moiety (20,21).

Butanolysis. Figure 4 shows the results of the butanolysis reactions for the six lipases using 1-butanol and isobutanol. For 1-butanol, comparable levels of monoesters were obtained for all six lipases after 2 h, but TLL-1 had produced the highest monoester content (78%) after 24 h (Fig. 4A). CAL did not show high activity, as in the ethanolysis and propanolysis reactions (only 54% after 24 h). The *Pseudomonas* lipases reached only 30–40% conversion into monoesters.

With the branched primary isobutanol, the situation changed substantially for some of the lipases as compared with the less bulky 1-butanol (Fig. 4B). The increase in monoester content was evident for RML, PCL, and CAL. This time, CAL displayed the highest yield, which after 24 h had increased dramatically from 54% for 1-butanol to 85%

for isobutanol. Significant increases also were observed for PCL and RML, but the activity of PFL remained as low as before, just above the 30% conversion level.

In general, CAL showed the most dramatic changes with respect to the type of butanols. The optimal butanol system for production of alkyl esters by enzymatic alcoholysis appears to be isobutanol with CAL, rather than 1-butanol.

Comparison of alcohols. In general, CAL showed the highest activity among all the lipases examined with most primary alcohols at a lower water content. For this lipase, higher yields were obtained with 1-propanol (93% after 24 h) than with methanol and ethanol (92%). Surprisingly, isobutanol (84%) was superior to 1-butanol as a substrate for CAL (54%). TLL-1 also reached relatively good yields under the reaction conditions studied. For TLL-1, the best alcoholic substrate was methanol, with 90% conversion after 24 h and 77–78% conversion with other straight-chain primary alcohols. 2-Propanol clearly reduced the activity of lipases less than isobutanol, which could relate to the higher polarity and larger steric hindrance of 2-propanol than isobutanol. RML, CAL, and PCL preferred isobutanol to 1-butanol; the remaining lipases did not discriminate strongly between these two alcohols.

Greater yields were obtained with 96% ethanol, which may also relate to the characteristics of the carrier. The highly hydrophilic property of the silica carrier of TLL-1 is probably the main reason, where enough water can be maintained in the surrounding lipase. In TLL-2, a hydrophobic polypropylene carrier was used for the same lipase. The activity was substantially lower in the latter case in general. PCL was strongly water dependent. The increase in water when replacing absolute ethanol with 96% ethanol increased the conversion dramatically, from 30 to 88%. RML was also affected by the amount of water, but not as significantly as PCL. RML showed a low preference for primary alcohols, but PCL definitely preferred isobutanol and longer-chain alcohols in general, which was again attributed to the influence of the water microenvironment of the lipase from different alcohols.

FFA content in alcoholysis. In general, a higher water content in lipase preparations resulted in a higher content of FFA as a result of hydrolysis side reactions (Table 1). The ethanolysis reaction involving CAL was an exception, with the FFA content being lower when 96% ethanol was used. This phenomenon was confirmed twice and the reason is not fully clear. It is probably due to a reduced overall enzyme activity, including hydrolysis activity, contradictory to the higher water content conditions. Another noticeable phenomenon relates to both TLL and the difference between 1-propanol and 2-propanol in terms of FFA formation, the latter alcohol resulting in a significantly higher FFA content. Isobutanol also resulted in a higher FFA content than 1-butanol for all the lipases studied. This could be because secondary or branched alcohols are more sterically hindered than primary alcohols. This renders these alcohols less nucleophilic, leading instead to a higher extent of hydrolysis.

ACKNOWLEDGMENTS

Support from the National Natural Science Foundation of China (20176020), the "863" High-Tech Project of China, and the Danish Technological Research Council (STVF) are acknowledged. The EU Major Research Infrastructure (MRI) program *via* the Center for Advanced Food Studies (LMC) is thanked for support for G.G.H.'s sabbatical at BioCentrum-DTU. Tiankui Yang and Lars Hellgren from BioCentrum-DTU are thanked for their technical help. Bert Nielsen is thanked for his practical assistance.

REFERENCES

1. Dossat, V., D. Combes, and A. Marty, Lipase-Catalyzed Transesterification of High Oleic Sunflower Oil, *Enzyme Microb. Technol. 30*:90–94 (2002).
2. Yahya, A.R.M., W.A. Anderson, and M. Moo-Young, Ester Synthesis in Lipase-Catalyzed Reactions, *Ibid*. *23*:438–450 (1998).
3. Krawczyk, T., Biodiesel, *inform 7*:801–830 (1996).
4. Breivik, H., G.G. Haraldsson, and B. Kristinsson, Preparation of Highly Purified Concentrates of Eicosapentaenoic Acid and Docosahexaenoic Acid, *J. Am. Oil Chem. Soc. 74*:1425–1429 (1997).
5. Bornscheuer, U.T. (ed.), *Enzymes in Lipid Modification*, Wiley-VCH, Weinheim, 2000.
6. Malcata, F.X. (ed.), *Engineering of/with Lipases*, Kluwer Academic, Dordrecht, 1996.
7. Hoy, C.-E., and X. Xu, Structured Triacylglycerols, in *Structured and Modified Lipids*, edited by F.D. Gunstone, Marcel Dekker, New York, 2001, pp. 209–240.
8. Shimada, Y., Y. Watanabe, T. Samukawa, and A. Sugihara, Conversion of Vegetable Oil to Biodiesel Using Immobilized *Candida antarctica* Lipase, *J. Am. Oil Chem. Soc*. *76*:789–792 (1999).
9. Selmi, B., and D. Thomas, Immobilized Lipase-Catalyzed Ethanolysis of Sunflower Oil in a Solvent-free Medium, *J. Am. Oil Chem. Soc. 75*:691–695 (1998).
10. Kaieda, M., T. Samukawa, A. Kondo, and H. Fukuda, Effect of Methanol and Water Contents on Production of Biodiesel Fuel from Plant Oil Catalyzed by Various Lipases in a Solvent-free System, *J. Biosci. Bioeng*. *91*:12–15 (2001).
11. Mittelbach, M., Lipase Catalyzed Alcoholysis of Sunflower Oil, *J. Am. Oil Chem. Soc*. *67*:168–170 (1990).
12. Shaw, J., D. Wang, and Y. Wang, Lipase-Catalyzed Ethanolysis and Isopropanolysis of Triglycerides with Long-Chain Fatty Acids, *Enzyme Microb. Technol*. *13*:544–546 (1991).
13. Lee, K.-T., T.A. Foglia, and K.-S. Chang, Production of Alkyl Ester as Biodiesel from Fractionated Lard and Restaurant Grease, *J. Am. Oil Chem. Soc*. *79*:191–195 (2002).
14. Schariter, J.A., J. Pachuski, B. Fried, and J. Sherma, Determination of Neutral Lipids and Phospholipids in the Cercariae of *Schistosoma mansoni* by High Performance Thin Layer Chromatography, *J. Liquid Chromatogr. Related Technol. 25*:1615–1622 (2002).
15. Dordick, J.S., Enzymatic Catalysis in Monophasic Organic Solvents, *Enzyme Microb. Technol*. *11*:194–211 (1989).
16. Koskinen, A.M.P., and A.M. Klibanov (eds.), *Enzymatic Reactions in Organic Media*, Blackie Academic and Professional, London, 1996.
17. Piyatheerawong, W., Y. Iwasaki, X. Xu, and T. Yamane, Dependency of Water Concentration on Ethanolysis of Trioleoylglycerol by Lipases, *J. Mol. Catal.–Enz. 28*:19–24 (2004).
18. Haraldsson, G.G., B.Ö. Gudmundsson, and Ö. Almarsson, The

Synthesis of Homogeneous Triglycerides of Eicosapentaenoic Acid and Docosahexaenoic Acid by Lipase, *Tetrahedron 51*:941–952 (1995).
19. Zhang, H., X. Xu, J. Nilsson, H. Mu, C.-E. Høy, and J. Adler-Nissen, Production of Margarine Fats by Lipase-Catalyzed Interesterification with a New Immobilized *Thermomyces lanuginosa* Lipase—A Large-Scale Study, *J. Am. Oil Chem. Soc. 78*:57–64 (2001).
20. Irimescu, R., K. Furihata, K. Hata, Y. Iwasaki, and T. Yamane, Utilization of Reaction Medium-Dependent Regiospecificity of *Candida antarctica* Lipase (Novozym 435) for the Synthesis of 1,3-Dicapryloyl-2-docosahexaenoyl (or -eicospentaenoyl) Glycerol, *J. Am. Oil Chem. Soc. 78*:285–289 (2001).
21. Halldorsson, A., C.D. Magnusson, and G.G. Haraldsson, Chemoenzymatic Synthesis of Structured Triacylglycerols by Highly Regioselective Acylation, *Tetrahedron 59*:9101–9109 (2003).

[Received December 6, 2004; accepted April 19, 2005]