Lipase-Catalyzed Production of Biodiesel[1]

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ABSTRACT: Lipases were screened for their ability to transesterify triglycerides with short-chain alcohols to alkyl esters. The lipase from *Mucor miehei* was most efficient for converting triglycerides to their alkyl esters with primary alcohols, whereas the lipase from *Candida antarctica* was most efficient for transesterifying triglycerides with secondary alcohols to give branched alkyl esters. Conditions were established for converting tallow to short-chain alkyl esters at more than 90% conversion. These same conditions also proved effective for transesterifying vegetable oils and high fatty acid-containing feedstocks to their respective alkyl ester derivatives.
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KEY WORDS: Alcoholysis, alkyl esters, biodiesel, grease, lipase, rapeseed, soy oil, tallow.

There have been a considerable number of studies that report transesterification and interesterification reactions by using lipases with and without organic solvents (1–6). Recently, research has centered on the use of lipases to transesterify higher-molecular weight fatty acids to alkyl esters. Lipase-catalyzed alcoholyses of sunflower oil (7), rapeseed oil (8), soybean oil, and beef tallow (9) have been reported. The alcoholysis reactions generally involve primary alcohols with a few scattered reports on transesterifications with secondary alcohols (10). Because of increasing environmental consciousness, the use of value-added products from agricultural fats and oils as biofuels has become important. Currently, rapeseed esters are used in Europe (11), and palm oil esters are being evaluated in Malaysia (12) as biodiesel. Soybean oil esters are featured prominently as potential diesel fuel alternatives (13), and there is a wide range of ongoing research in this area. Methyl and ethyl tallowates have been tested as diesel fuel substitutes (14–18). In light of the high price of soybean oil-derived biodiesel, relative to petrodiesel, at the present time, extending soybean oil feedstock with tallow—a cheaper feedstock—would be advantageous. A major drawback in the use of neat tallow esters, however, is their cold-temperature properties when compared to soy or petroleum diesel fuel. Blending tallow esters with soy esters would improve these cold temperature properties. Another way of improving cold-temperature properties of tallow esters would be to substitute methanol with branched higher-molecular weight alcohols.

Though efficient in terms of reaction yield and time, the chemical approach to synthesizing alkyl esters (18–20) from triglycerides has drawbacks, such as difficulties in the recovery of glycerol, the need for removal of salt residue, and the energy-intensive nature of the process. On the other hand, biocatalysts allow for synthesis of specific alkyl esters, easy recovery of glycerol, and transesterification of glycerides with high free fatty acid (FFA) content. This technology could be extended to transesterification of greases, which are even less expensive than soybean oil and tallow. This process can further be used to synthesize other value-added products, including biodegradable lubricants and additives for fuel and lubricants. Lipase can also be used to introduce other functionalities into alkyl esters that may further improve the cold-temperature properties of the resulting biodiesel. In this paper, we report the lipase-catalyzed synthesis of normal and branched-chain alkyl esters of agriculturally derived triglycerides (TG): vegetable oils, tallow, and restaurant grease.

MATERIALS AND METHODS

Materials. Tallow was obtained from Chemol Corp. (Greensboro, NC), and high free fatty acid-containing greases ("restaurant" or "yellow" grease) were donated by Kaluzny Bros. (Joliet, IL). Rapeseed oil was donated by Calgene Chemical (Skokie, IL). Soybean and olive oils were purchased from a local supermarket.

Supported lipases used in this study were preparations of *Mucor miehei* (Lipozyme IM60) and *Candida antarctica* (SP435), both from Novo Nordisk Bioindustrials (Franklinton, NC). Lipase powders from *Geotrichum candidum* and *Pseudomonas cepacia* (PS30) were from Amano Pharmaceutical (Troy, VA), and *Rhizopus delemar* was from Seikagaku Kogyo Co. (Tokyo, Japan).

All solvents were of high-performance liquid chromatography (HPLC) grade and were purchased from Burdick and Jackson (Muskegon, MI). *N,O*-(bistrimethylsilyl) trifluoroacetamide (BSTFA) was obtained from Regis Chemical Co. (Morton Grove, IL). Unless otherwise stated, all other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Methods. Screening experiments with lipases were conducted at 10% lipase by weight of TG, with hexane as sol-

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vent. A typical reaction was run as follows: to a stoppered 125-mL Erlenmeyer flask, containing tallow (2.74 g, 2.88 mmol) in hexane (7.7–8.15 mL), was added 3 mole equivalents of the alcohol and the appropriate amount of enzyme. The reaction mixture was shaken at 200 rpm for 5 h at 45°C. Progress of the reaction was followed by taking 100-μL aliquots at selected time intervals and concentrating to a residue, which was derivatized with BSTFA for gas-chromatographic analysis. Other reactions were run in a similar manner but without solvent.

Analysis of products. For solvent reactions, an aliquot was taken at selected time intervals and freed of solvent under a stream of nitrogen at 45°C. A portion of the residue (10 mg) was dissolved in tetrahydrofuran (100 μL), and BSTFA (200 μL) was added. The mixture was heated on a water bath at 90–95°C for 15 min. After cooling the mixture to room temperature, hexane (5 mL) was added. An aliquot of 0.5 μL of the mixture was separated by lipid class (alkyl ester, monoglyceride, diglyceride, and TG) according to carbon number (21) by gas chromatography as follows: a 15-m long, nonpolar high-temperature capillary column (DB1-HT), 0.32 mm i.d., 0.1 μm film thickness was used (J&W Scientific, Folsom, CA) for analyses. The silylated samples were injected directly on-column into a Hewlett-Packard (Avondale, PA) 5890 gas chromatograph, at a helium carrier gas flow rate of 5.5 mL/min, with flame-ionization detection. The initial oven temperature was 70°C, followed by a temperature program of 20°C/min to a final temperature of 350°C, which was held for 4 min. Peaks in the chromatograms were identified by comparison of retention times with standards of known compositions.

RESULTS AND DISCUSSION

Using Mittelbach's (7) conditions with hexane as solvent, we screened commercially available lipases for their abilities to transesterify the TG of olive, soybean oil, and tallow with short-chain alcohols to their alkyl ester derivatives. The enzymes studied included a 1,3-specific (22) (*M. miehei*), an acyl-specific (23) (*G. candidum*), and a nonspecific (22) (*P. cepacia*) lipase. For methanolysis, the lipase from *M. miehei* (Lipozyme™ IM60) was the most effective in converting olive, soybean, and tallow to the corresponding methyl ester derivatives (Table 1). When the same reaction conditions (45°C, 5 h reaction) were used

TABLE 1
Lipase-Catalyzed Transesterification of Triglycerides to Alkyl Esters with Primary Alcohols[a]

Substrate	Alcohol	Lipase	Temperature (°C)	% Composition of product[b,c] MG	DG	TG	Ester
Tallow	Methanol	*Mucor miehei*[d]	45	0.5 e	8.2 e	13.6 f	77.8 b
Tallow[e]	Methanol	*M. miehei*	45	0.1 e	3.5 f–i	1.5 g	94.8 a
Soybean	Methanol	*M. miehei*	45	1.4 e	12.5 d	10.7 f	75.4 b,c
Rapeseed	Methanol	*M. miehei*	45	1.9 d,e	7.8 e,f	13.0 f	77.3 b
Tallow	Methanol	*Candida antarctica*[d]	45	5.1 c	12.8 d	53.5 d	25.7 d
Tallow	Methanol	*Pseudomonas cepacia*	45	0.0 e	6.9 e,f,g	79.2 b	13.9 e,f
Soybean	Methanol	*P. cepacia*	45	2.4 d,e	17.8 c	65.3 c	14.5 e,f
Olive	Methanol	*P. cepacia*	45	1.3 e	24.2 a,b	50.1 d	24.4 d
Tallow	Methanol	*Rhizopus delemar*	45	0.2 e	4.1 e-i	95.09 a	0.8 g
Olive	Methanol	*R. delemar*	45	0.2 e	3.1 g,h,i	96.1 a	0.6 g
Soybean	Methanol	*R. delemar*	45	0.2 e	3.9 e–i	95.0 a	0.8 g
Tallow	Methanol	*Geotrichum candidum*	45	6.3 c	3.7 f–i	77.5 b	12.5 e,f
Tallow	Ethanol	*M. miehei*	45	0.1 e	0.9 h,i	0.7 g	98.3 a
Tallow	Ethanol[f]	*M. miehei*	45	14.4 b	22.4 b	1.6 g	68.0 f,g
Tallow	Ethanol	*M. miehei*	35	0.0 e	4.6 e-h	1.4 g	93.9 a
Tallow	Ethanol	*M. miehei*	55	0.4 e	3.3 g,h,i	1.8 g	94.5 a
Soybean	Ethanol	*M. miehei*	45	0.6 e	1.2 h,i	0.8 g	97.4 a
Rapeseed	Ethanol	*M. miehei*	45	0.8 e	0.3 h,i	0.3 g	98.2 a
Tallow	Ethanol	*P. cepacia*	45	17.6 a	15.7 c,d	52.7 d	13.7 e,f
Tallow	Ethanol	*R. delemar*	45	4.3 c,d	28.5 a	46.0 d,e	21.2 d,e
Tallow	Propanol	*M. miehei*	45	0.2 e	1.5 h,i	0.1 g	98.3 a
Tallow	Propanol[f]	*M. miehei*	45	0.7 e	0.5 h,i	0.3 g	98.6 a
Tallow	Butanol	*M. miehei*	45	0.1 e	0.1 i	0.2 g	99.6 a
Tallow	Butanol[f]	*M. miehei*	45	0.6 e	0.5 h,i	0.8 g	98.1 a
Tallow	Isobutanol	*M. miehei*	45	0.1 e	0.8 h,i	0.8 g	98.5 a
Tallow	Isobutanol[f]	*M. miehei*	45	0.2 e	0.2 i	0.2 g	99.4 a
Tallow	Isobutanol	*P. cepacia*	45	6.8 c	27.1 a	37.3 e	28.8 d
Tallow	Isobutanol	*R. delemar*	45	0.6 e	16.3 c,d	72.7 b,c	10.4 f

[a]Reaction conditions for transesterification were as follows: 0.34 M triglyceride in hexane (8 mL), 200 rpm, 5 h reaction time; MG, monoglyceride; DG, diglyceride; TG, triglyceride.
[b]Determined by gas chromatography.
[c]Means ($n = 3$) in the same column with no letter in common are significantly different ($P < 0.05$) by Bonferroni least significant difference.
[d]*Mucor miehei* IM60 and *C. antarctica* SP 435 (Novo Nordisk Bioindustrials, Franklinton, NC).
[e]Reaction time was 8 h.
[f]Water, 6.0 mol% based on triglyceride, was added to reaction.

with ethanol and isobutyl alcohol with tallow as the TG, the lipase of Lipozyme IM60 also was most effective in converting tallow to the respective alkyl esters (Table 1). Thus, lipase from *M. miehei* was most efficient for transesterification of TG with primary alcohols to alkyl esters for the series of methanol to isobutyl alcohol. To increase conversions when methanol was the alcohol, a reaction time of 8 h was needed. The conditions used for high conversions with methanol were sensitive to the amount of water added to the reaction mixtures, with water greatly reducing the amount of ester formed. The same was true for ethanol. The use of 95% ethanol instead of absolute ethanol gave conversions that dropped from 98 to 68% (Table 1). Propanol, butanol, and isobutyl alcohol were prepared with 12.5 wt% enzyme at an alcohol-to-tallow ratio of 3:1. Water did not appear to affect ester production in these instances. The conversions were practically constant over temperature ranges between 35–55°C, as exemplified by ethanolysis of tallow (Table 1).

Screening reactions for transesterification of tallow with secondary alcohols (Table 2) showed a completely different trend, in which the lipases from *C. antarctica* and *P. cepacia* gave higher ester conversions than Lipozyme IM60. This is in agreement with the findings of Shaw and Wang (10), who found immobilized *P. cepacia* to be effective in the ethanolysis/isopropanolysis of tripalmitin and triolein. For production of esters from secondary alcohols, the amount of enzyme used was 25 wt% based on TG. Reactions run without the addition of water were sluggish for both SP 435 and PS30 lipases. Maximum conversion of 60–84% was obtained overnight (16 h). With the addition of small amounts of water (3–100 μL), improved conversions were obtained. The need for water to improve conversions also was supported by the ease with which high FFA-containing greases were converted to their corresponding branched alkyl esters. The opposite effect was observed for methanolysis, which was extremely sensitive to the presence of water. For branched-chain alcohols, better conversions were obtained when the reactions were run neat, as seen with isopropanol and 2-butanol (Table 3). Lower yields with methanol and ethanol in solvent-free reactions could be attributed to unfavorable viscosity conditions, which affect intimate mixing of substrates with the lipase.

The conditions established for tallow (0.34 M tallow in hexane, 45°C, 200 rpm, 4–8 h, 12.5–25% enzyme by weight of tallow, alcohol-to-TG ratio of 3:1, no hexane for branched alcohols) have been used to scale-up (120 g of TG) reactions involving the primary alcohols to give over 95% conversions, and over 90% for secondary alcohols, with minimum production of other glycerides (Table 3). These conditions also gave conversions between 75–85% when applied to soybean and rapeseed oils, as exemplified by methanolysis and isopropanolysis reactions (Fig. 1). When applied to greases with varying fatty acid contents, methanolysis was curtailed for feedstocks where the FFA content was greater than 9%, while ethanolysis was effective below 22.4% FFA. Secondary alcohols were extremely effective in converting high fatty acid-containing feedstocks to their respective alkyl esters (Fig. 2). This is in agreement with our earlier observation that water appears to retard the conversion to ester when methanol is the substrate but promotes ester formation when secondary alcohols are used with *C. antarctica* as the lipase.

In conclusion, lipase esterification is a viable method for the production of alkyl esters from tallow, vegetable oil, and greases. Work is still ongoing to maximize conversions for specific alcohols, to improve conversions for solvent-free methanolysis and ethanolysis, to scale-up reactions to provide sufficient quantities for determining their cold-temperature properties and to further improve upon these properties, emis-

TABLE 2
Lipase-Catalyzed Transesterification of Tallow to Alkyl Esters with Secondary Alcohols[a]

Alcohol	Solvent	Lipase	Temperature (h)	% Composition of product[b,c]			
				MG	DG	TG	Ester
Isopropanol	Hexane	*Candida antarctica*[d]	5	0.8 b	8.7 d	49.3 b	41.2 d
Isopropanol	Hexane	*Pseudomonas cepacia*	5	5.2 a	24.7 b	26.0 c	44.1 d
Isopropanol	Hexane	*Mucor miehei*[d]	5	7.4 a	14.3 c	54.0 b	24.3 e
Isopropanol	Hexane[e]	*C. antarctica*	5	2.1 b	5.2 d,e	31.5 c	61.2 b
Isopropanol	Hexane	*C. antarctica*	16	0.0 b	1.1 f	47.2 b	51.7 c
2-Butanol	Hexane	*C. antarctica*	5	0.2 b	1.9 e,f	74.2 a	23.7 e
2-Butanol	Hexane	*P. cepacia*	5	0.3 b	29.7 a	29.0 c	41.0 d
2-Butanol	Hexane	*M. miehei*	5	2.4 b	23.2 b	54.3 b	19.6 e
2-Butanol	Hexane[e]	*C. antarctica*	5	6.2 a	8.3 d	49.6 b	39.0 d
2-Butanol	Hexane	*C. antarctica*	16	0.0 b	1.1 f	15.4 d	83.8 a

[a]Reaction conditions were as follows: 0.34 M tallow in hexane (8 mL), 45°C, 0.3 g of enzyme, 200 rpm. See Table 1 for abbreviations and company source.

[b]Determined by gas chromatography.

[c]Means ($n = 3$) in the same column with no letter in common are significantly different ($P < 0.05$) by Bonferroni least significant difference.

[d]*Mucor miehei* IM60 and *C. antarctica* SP435.

[e]Water added at 6 mole% based on tallow.

TABLE 3
Lipase-Catalyzed Transesterification of Tallow with Alcohols[a]

Alcohol	Solvent	Lipase	Temperature (h)	% Composition of product[b,c] MG	DG	TG	Ester
Methanol	Hexane	*Mucor miehei*[d]	5	0.5 c,d	8.2 b	13.6 d	73.8 d
Methanol	Hexane	*M. miehei*	8	0.1 d	3.5 c	1.5 e	94.8 a
Methanol	None[e]	*M. miehei*	8	5.2 a	10.0 a	67.6 a	19.4 g
Ethanol	Hexane	*M. miehei*	5	0.2 d	1.2 d	0.6 e	98.0 a
Ethanol	None[e]	*M. miehei*	5	1.8 b,c	3.7 c	29.0 c	65.5 e
Isopropanol	Hexane	*Candida antarctica*[d]	16	0.0 d	1.1 d	47.2 b	51.7 f
Isopropanol	None[e]	*C. antarctica*	16	2.2 b	7.0 b	0.9 e	90.3 b
Isobutanol	Hexane	*M. miehei*	5	0.1 d	0.8 d	0.6 e	98.5 a
Isobutanol	None[e]	*M. miehei*	5	0.8 c,d	0.9 d	1.0 e	97.4 a
2-Butanol	Hexane	*C. antarctica*	16	0.0 d	1.1 d	15.4 d	83.8 c
2-Butanol	None[e]	*C. antarctica*	16	1.3 b,c,d	1.3 d	1.0 e	96.4 a

[a]Reaction conditions for transesterification were as follows: 0.34 M tallow in hexane (8 mL), 45°C, 200 rpm. See Table 1 for abbreviations.

[b]Determined by gas chromatography.

[c]Means ($n = 3$) in the same column with no letter in common are significantly different ($P < 0.05$) by Bonferroni least significant difference.

[d]*Mucor miehei* IM60 and *C. antarctica* SP435.

[e]Reaction conditions the same except no solvent used.

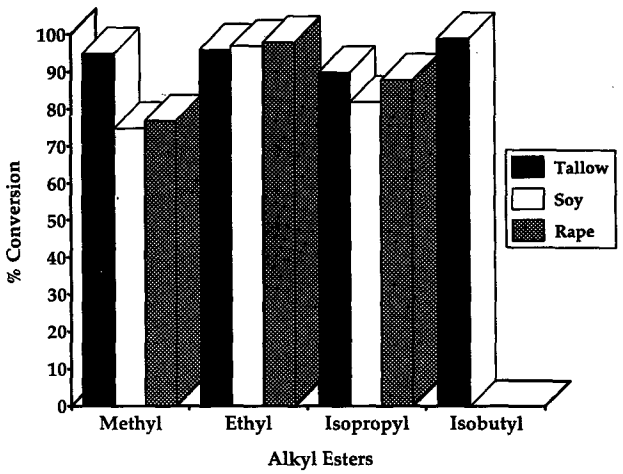

FIG. 1. Lipase-catalyzed transesterification of fats and oils with primary (*Mucor miehei*) and secondary (*Candida antarctica* lipase) alcohols; FFA, free fatty acids.

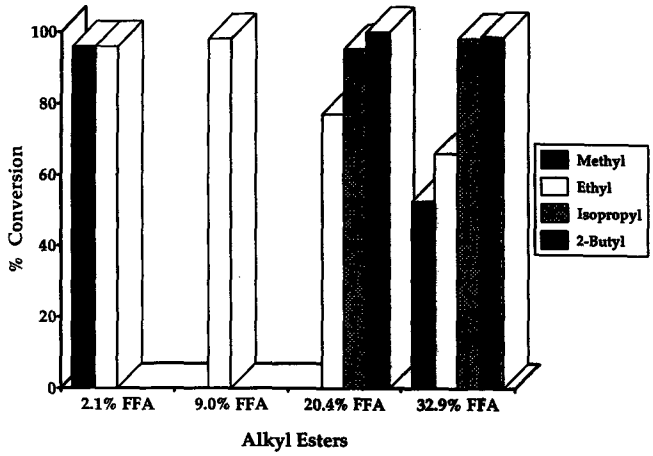

FIG. 2. Enzymatic transesterification and esterification of FFA-containing triglyceride feedstocks using *Mucor miehei* (1° alcohols) and *Candida antarctica* (2° alcohols) lipases. See Figure 1 for abbreviations.

sion, and performance characteristics of the alkyl esters as diesel fuel alternatives. The potential of this technology is also being explored to introduce other functionalities to prepare biodegradable lubricants and additives.

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