6343

Combined Nonenzymatic–Enzymatic Method for the Synthesis of Simple Alkyl Fatty Acid Esters from Soapstock

Michael J. Haas* and Karen M. Scott

USDA, ARS, Eastern Regional Research Center, Wyndmoor, Pennsylvania 19038

ABSTRACT: Soapstock (SS) is a by-product of the extraction of oilseeds to produce edible oils. Annual U.S. production exceeds one-half million tons. A representative sample of SS consists of 45.1% water, 10.0% free fatty acids, 10.1% triglycerides, 1.8% diglycerides, 3.6% phosphatidylethanolamine, 2.2% phosphatidylinositol, 2.7% phosphatidylcholine, 14.0% solvent-insolubles and 10.5% other material, which was not characterized. A process has been developed that sequentially employs a nonenzymatic and an enzymatic step to convert the lipid-linked and the free fatty acids of SS to the esters of monohydric alcohols. The first step of the process employed alcohol and potassium hydroxide to transesterify the glyceride- and phosphoglyceride-linked fatty acids of the substrate. Because water inhibited the reaction, it was necessary that the SS be dried before use. Nonetheless, even with some batches of SS with water contents below 1% (weight basis), ester hydrolysis accompanied esterification. Each of five examined simple primary alcohols participated effectively in the transesterification reaction, which proceeded rapidly at room temperature and was essentially complete within 1 h. The average ratio of transesterification to hydrolysis in four examined small primary alcohols was 4:1. However, in methanol this value was 99:1 due to the virtual absence of hydrolysis. Significant transesterification by a secondary alcohol (isopropanol) did not occur at room temperature. The minimum effective molar ratio of alcohol to lipid-linked fatty acids was 20:1. The minimum effective concentration of KOH was between 0.10 and 0.15N. The efficiency of the transesterification reaction exceeded 90% of theoretical maximum. The second step of the process involves lipase-mediated esterification of the free fatty acids in the preparation that are not esterified by the alkaline transesterification. Of four lipase preparations examined (Novo Lipozyme IM 20 and SP435, and Amano PS30 and CE), only SP-435 catalyzed significant esterification of the free fatty acids. The reaction was not catalyzed by heat-denatured enzyme. In the pH range between 6 and 13.5, the enzyme reaction proceeded best at pH 6, although also well at pH 7. The optimal water concentration was 0.70% (vol/vol). At an enzyme dosage of 1.1% (weight basis, relative to the dry weight of SS present) under optimal conditions and at 42°C, 63% of the free fatty acids in a post-alcoholysis mixture were enzymatically esterified. The addition of molecular sieves did not increase esterification, which was probably retarded by the high viscosities of the reactions. Under the optimal conditions identified here, the degree of conversion of the fatty acids in SS to simple alkyl esters by the combined reaction scheme was 81%. Opportunities exist for further optimization of these reactions.

JAOCS 73, 1393–1401 (1996).

KEY WORDS: Alcoholysis, esterification, fatty acid ester, lipase, phospholipid, soapstock, transesterification, triglyceride.

The production of edible vegetable oils generates an intermediate crude oil that contains not only triglycerides but also partially substituted glycerides, phosphoglycerides, free fatty acids and minor components, such as sterol esters and pigments. All these must be removed from the triglyceride fraction to produce a stable, acceptable edible oil. One step in the refining process involves the addition of water and alkali, causing precipitation of a semisolid material known as soapstock (SS), which contains not only the salts of the free fatty acids but also some residual triglycerides, mono- and diglycerides, phosphoglycerides, pigments, some of the lesser components of the crude oil, and water (1). From soybeans, the predominant source of edible oil in the United States, SS is generated at a rate of about 6% of the volume of crude oil produced (2), amounting to approximately one billion pounds annually. Edible oil production from other vegetable sources also generates considerable, though smaller, quantities.

Although it has found use as a component of animal feeds, as a source of free fatty acids and low-grade oil for industrial use, and as a topcoating to reduce dust on gravel roads, there remains interest in the development of new uses for soapstock. The simple alkyl esters of fatty acids exhibit lubricant, solvent, and surface-active properties. Therefore, they are used in large quantities in numerous industrial applications, including cosmetics, textiles, and metalworking. In addition, they are the primary feedstocks for the production of fatty alcohols and are being investigated as fuels for diesel engines. We have therefore explored the possibility of developing a process for the synthesis of simple fatty acid esters from SS. The goal was the development of a method that would produce esters not only from the free fatty acids in SS but also from the fatty acids found in its polar and nonpolar lipid fractions. Such a process would create new outlets for SS, could provide fatty acid esters at attractive costs (particularly in comparison to esters presently derived from refined triglyc-

*To whom correspondence should be addressed.

erides), and would produce glycerol, a useful and marketable material, as a by-product.

For present purposes, the fatty acids of SS can be classified into two groups: (i) those that are esterified to other molecules, forming the glyceride, phosphoglyceride, and other components of SS, and (ii) free, unesterified fatty acids. The reaction whereby fatty acids in the former group are transferred to simple monohydric alcohols, a type of transesterification, is termed "alcoholysis." This reaction can be catalyzed either enzymatically (3–7) or nonenzymatically (8–13) and has been extensively studied. Nonenzymatic methods have been studied for several decades and are attractive for their speed, simplicity, and economy. The simplest of these involve the mixing of feedstock and alcohol under alkaline conditions at ambient or slightly elevated temperatures. Although there have been some studies with SS (13), the majority of the published work on transesterification has dealt with refined triglycerides as the feedstock.

Free fatty acids, the other fatty acid class in SS, are not esterified under the alkaline conditions that successfully esterify lipid-linked fatty acids. Their esterification can be catalyzed, however, by lipases (triacylglycerol acylhydrolase, EC 3.1.1.3). Although the enzymatic esterification of fatty acids has often been studied in alkane solvents (14,15), it also has been demonstrated to occur directly in solutions that contain only alcohol and free fatty acid (6,7,15–17). Due to the simplicity of this latter approach, we chose to apply it to the esterification of the free fatty acids remaining in SS solutions after alkaline transesterification.

In current industrial practice, the acidulation of SS generates a fraction, known as acid oil, that contains protonated free fatty acids (40% to >85%), neutral glycerides, and unsaponifiables (18). Stern *et al.* (19) described a multistep nonenzymatic process, conducted at temperatures of 100 to 120°C, to esterify the fatty acids in acid oils. However, lipid-linked fatty acids were not esterified. The Twitchell process can be used to split and esterify acidulated soapstocks, but this process is lengthy and energy-intensive (8). The simultaneous lipase-catalyzed esterification of the fatty acids and transesterification (alcoholysis) of the glyceride-bound fatty acids of acid oils also have been recently described (16). We reasoned that a nonenzymatic approach to the transesterification portion of this reaction scheme offered advantages of speed and simplicity over enzyme catalysis. In addition, a method capable of using SS itself as the feedstock, rather than acid oil derived from SS, would eliminate the acidulation step and could increase the overall conversion efficiency because not all lipid-linked fatty acids of a soapstock necessarily appear in the acid oil produced from it. For these reasons, we chose to investigate the potential of alkali-catalyzed transesterification followed by lipase-catalyzed esterification to synthesize the alkyl esters of SS fatty acids.

EXPERIMENTAL PROCEDURES

Chemicals. L-α-Phosphatidylcholine, L-α-phosphatidylethanolamine and L-α-phosphatidylinositol (all from soybeans and all with purities in excess of 99%) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Soybean oil, diglyceride, and free fatty acids for use as analytical standards were from Sigma Chemical Co. (St. Louis, MO). For quantitating fatty acid levels, a mixture of fatty acids was made whose composition reflected that of soybean oil. A fatty acid methyl ester mixture (RM-1; Matreya, Inc., Pleasant Gap, PA) whose fatty acid composition also was comparable to that of soybean glycerides was used as the standard for quantitating fatty acid esters. Lipozyme IM 20 (immobilized *Rhizomucor miehei* lipase) and SP-435 (immobilized *Candida antarctica* B lipase) were the products of Novo Nordisk BioChem (Franklinton, NC). Lipases CE (from *Humicola lanuginosa*) and PS30 (*Pseudomonas* sp.), supplied as dry powders, were gifts from Amano Enzyme U.S.A. Co., Ltd. (Troy, VA). Ethanol (U.S.P., 200 proof, anhydrous) was produced by the Warner-Graham Co. (Cockeysville, MD). Methanol, isopropanol, and hexane (Burdick and Jackson Brand) were purchased from Baxter (Muskegon, MI). Isoamyl alcohol and 1-butanol (Baker Analyzed Reagents) were obtained from J.T. Baker Inc. (Phillipsburg, NJ). Isobutyl alcohol (AR) was produced by Mallinkrodt Inc. (Paris, KY). *Bis*(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Regis Chemical Co. (Morton Grove, IL). Tetrahydrofuran (THF) and molecular sieves (3Å) were the products of Aldrich Chemical Co. (Milwaukee, WI). The sieves were activated just prior to use by a 17-h incubation at 75°C. SS from soybean processing was a gift from Cargill (Minneapolis, MN) and was stored at –20°C until use.

SS analysis. To determine the water content of SS, preweighed samples were lyophilized to constant mass, and their residual weights were measured. To determine the free fatty acid and glyceride contents, a sample was adjusted from its normal pH of 10.5 to 2 with H_2SO_4 and dissolved in a 40-fold (vol/wt) excess of hexane. The solution was centrifuged ($3500 \times g$, 25 min) in preweighed tubes. After removal of the resulting liquid layer, the solid residue was dried and weighed, and the amount of solvent-insoluble material was calculated. The liquid layer was filtered over Millex-FH_{13} filters (Millipore, Bedford, MA) and analyzed by high-performance liquid chromatography (HPLC). To quantitate the amounts of phospholipids, SS was adjusted to pH 2 with 5N H_2SO_4, dissolved in a 40-fold (vol/wt) excess of chloroform/methanol (85:15, vol/vol), centrifuged as described above to remove insoluble material, and filtered through Millex-HV_{13} filters (Millipore). Samples were then analyzed by HPLC.

Alkaline transesterification of lipid-linked fatty acids in SS. A magnetic stirring bar or orbital shaker was employed to mix SS in alcohol overnight at room temperature in a sealed tube. When a single-phase solution did not result from this treatment, a spatula was employed to further pulverize the substrate. Stock solutions of potassium hydroxide in alcohol were prepared just prior to use. Transesterification was initiated by adding an aliquot of the KOH solution, as indicated

in the text, to the SS-alcohol solution and stirring at room temperature. To determine the degree of reaction, aliquots were neutralized with 5N HCl, and their alcohol was removed with a stream of nitrogen. The residue was resuspended in ethanol/water (9:1, vol/vol), acidified with 5N HCl (<pH 3.5) to protonate free fatty acids, and extracted with hexane. The hexane extract was filtered through Millex-FH_{13} filters and analyzed by HPLC.

A proportion of the solids in SS is insoluble in hexane. To the extent that this insoluble fraction contributes to ester production during transesterification, it would interfere with the calculation of theoretical yields of ester, which were based solely on the content of hexane-soluble lipids in SS. Therefore, it was determined whether this material could serve as a substrate for the formation of esters in the transesterification reaction. A sample (0.6 g) of SS was acidified as described in *SS analysis* of the Experimental Procedures section of this paper and dissolved in 24 mL hexane, and the insoluble fraction was isolated as described in *SS analysis*. This material was transesterified by incubation for 2 h in 2.5 mL of 0.12N KOH in ethanol. The sample was then acidified and hexane-extracted, and its ethyl ester content was determined by HPLC.

Enzymatic esterification of the free fatty acids in SS. After alkaline transesterification, the pH values of entire reaction mixtures were adjusted to desired values, as indicated in the text, with concentrated HCl. In investigations of the water requirement of the enzymatic step, water was added at this time, followed by lipase. After incubation at 42°C, the mixture was acidified (<pH 3.5) with concentrated HCl, one-fourteenth volume of water was added, and the solution was extracted with 6 vol of hexane. Aliquots of the organic phase were filtered through Millex-FH_{13} filters, and the free fatty acid content was determined by HPLC.

HPLC. The lipid components of reaction mixtures were separated by HPLC on a Lichrosorb Si 60-5 column (3 × 100 mm; Chrompack, Inc., Raritan, NJ) that was eluted with gradients of isopropanol and water in hexane/0.6% acetic acid (20). Peaks eluting from the column were detected with an evaporative light-scattering mass detector (ELSD IIA; Alltech, Deerfield, IL), operating at a nebulizer temperature of 60°C and a nitrogen flow rate of 3.5 L/min. Peaks were identified and quantitated by comparison of their retention times and areas to those of known standards. With this system, it was possible to detect and quantitate free fatty acids, glycerides, and individual phosphoglycerides at levels less than or equal to 0.5, 0.5, and 10%, respectively, of their amounts in pretransesterification mixtures.

The total glyceride- and phosphoglyceride-bound fatty acid content at the start of the reactions, here termed the "transesterifiable fatty acid" content, was calculated from the compositional data determined for SS. The ester contribution from the unidentified materials of the hexane-insoluble material in SS was also included in the calculation of the "transesterifiable fatty acid content" value. Extents of fatty acid ester synthesis by alkaline transesterification are expressed as percentages of this value.

The substrate for enzymatic esterification was always an alkaline transesterification mixture that had been conducted under optimum conditions. Because the transesterification reaction converted the majority of the detectable lipid-linked fatty acids to alkyl esters and free fatty acids, only free fatty acids remained to serve as substrates for lipase-catalyzed esterification. Therefore, enzyme activities are expressed as the degree of reduction in free fatty acid content. These reductions were always accompanied by proportionate increases in the fatty acid ester content.

All data are the results of duplicate independent determinations.

Gas-chromatographic analysis of post-transesterification reactions. Gas chromatography (GC) was used to verify that the prominent new HPLC peak formed during transesterification of SS was composed of fatty acid esters. Two grams of SS were stirred overnight in 9 mL ethanol. Three-and-one-half mL of a fresh solution of KOH in ethanol (0.02 g/mL) was added. Following a 30-min incubation at room temperature, 1.55 mL water was added, and the mixture was extracted four times with an equal volume of hexane. One mL of the pooled hexane extracts was concentrated to dryness. Two-hundred microliters of BSTFA and 100 μL of THF were added, and the mixture was heated for 15 min at 95°C. The sample was diluted with hexane and analyzed by GC *via* direct on-column injection onto a nonpolar high-temperature capillary column (DB1-HT, 15 m × 0.32 mm i.d., film thickness 0.1 micron; J&W Scientific, Folsom, CA). The carrier gas was helium, flowing at 5.5 mL/min. The initial oven temperature of 70°C was raised at a rate of 20°C/min to 350°C and held for 4 min. Compounds were detected by flame-ionization and identified by reference of their retention times to those of various fatty acid ethyl esters. This treatment would neither esterify free fatty acids in the preparation nor transesterify glyceride- or phosphoglyceride-bound fatty acids. It was designed solely to allow detection of preformed ethyl esters by GC.

Inactivation of SP-435. A suspension of 0.5 g enzyme in 10 mL distilled water was boiled for 5 min. After cooling to room temperature, the resin was recovered by filtration, washed twice by resuspension in 40 mL distilled water and finally by resuspension in 40 mL ethanol. The resin was recovered by filtration and blown dry with nitrogen. Final traces of liquid were removed under vacuum. The amount of activity remaining after this treatment was quantitated with a titrimetric lipase assay (pH 7.5) with emulsified olive oil as the substrate (21).

RESULTS AND DISCUSSION

Compositional analysis of SS. As received, SS contained 45.1% water. The major lipids detected by HPLC analysis of hexane and of chloroform/methanol extracts of SS were triglycerides (10.1% content, wet weight basis, 18.3% dry weight basis), diglycerides (1.8%, 3.3%), phosphatidylethanolamine (3.6%, 6.6%), phosphatidylcholine (2.7%,

5.0%), phosphatidylinositol (2.2%, 4.0%), free fatty acids (10.0%, 18.2%), other solvent soluble materials not identified (10.5%, 19.1%) and solvent-insoluble material (14.0, 25.5%). These data were used to calculate the amounts of fatty acid present in each major component of SS and the total amount of free and lipid-linked fatty acid in the sample. By using the fatty acid composition of soy oil, a value of 274.1 was calculated for the molecular weight of an average soy fatty acid. Based on the above data, it was then calculated that the total lipid-linked fatty acids constituted 1.088 mmoles per gram dry weight of SS and that the free fatty acid content was 0.664 mmoles per gram dry weight. Among the components not quantitated were monoglycerides (estimated to comprise less than 4% of the total solids), sterols, acylglucosides, minor-frequency phospholipids, and salts and other solvent-insolubles. Little esterifiable fatty acid was located in the hexane-insoluble material: transesterification of this fraction produced only 0.031 mmole of ester per gram dry weight of SS.

Alkali-catalyzed transesterification of SS. The utility of nonenzymatic catalysis in the transesterification of the glyceride components of SS was investigated because it is simple, rapid, and inexpensive. Alkaline, rather than acidic, catalysis was studied because the former reaction proceeds more rapidly, can be conducted with simpler equipment, and is more suited for processing an alkaline feedstock such as SS. Potassium hydroxide, rather than the more commonly used sodium hydroxide, was used due to the possibility of subsequent use of the potassium-rich wastes as fertilizer, an option not available for wastes rich in sodium ion.

Despite reports involving the use of reflux conditions for the alkaline transesterification of triglycerides in alcohol (11), the reaction occurs relatively rapidly at temperatures near ambient. (Higher temperatures do, however, increase the fluidity of the mixture and facilitate the recovery of the glycerol.) Thus, Freedman *et al.* (12) reported ester yields of 99% after a 5-h incubation of soybean oil in alkaline methanol at 32°C. We have found that alkaline transesterification of SS proceeds well under reflux conditions (data not shown). However, because it also proceeds rapidly without heat, the majority of our investigations were conducted at room temperature. Initial experiments demonstrated that alcoholic KOH catalyzed rapid transesterification of the glyceride- and phosphoglyceride-linked fatty acids of lyophilized SS. HPLC analysis of some posttransesterification reactions demonstrated the complete absence of tri-, di-, and monoglycerides and all phospholipid components of SS and the appearance of a prominent peak with a retention time that is characteristic of fatty acid alkyl esters. Gas-chromatographic analysis confirmed that, for the reaction between ethanolic KOH and SS, this new product was composed exclusively of fatty acid ethyl esters and that ester production was accompanied by the disappearance of the glycerides in the sample.

Effect of the water content of SS on the transesterification reaction. Because the alkaline transesterification reaction is known to be inhibited by water, samples of SS were dried to various extents by lyophilization, and the transesterification efficiency was determined at each level. Transesterification by either ethanol or isobutanol was depressed by water (Fig. 1). Ester production fell markedly, even when using SS with a residual water content of 18%, the lowest nonzero value examined (Fig. 1). Although it is possible that small amounts of water may be tolerated by this reaction, all subsequent experiments reported here used lyophilized SS with a residual water content of less than 0.6%.

Effect of the identity of the alcohol on the transesterification reaction. To determine the generality of the alkaline transesterification reaction with regard to the alcohol co-substrate, the reaction was conducted in each of several simple monoalcohols. Reaction mixtures initially contained 1.2 g of dry SS in a final volume of 9.2 mL of alcohol that contained KOH at a final concentration of 0.11N. To reduce potential inhibition by water, the alcohols were predried by overnight shaking with 5% (wt/vol) anhydrous sodium sulfate. Sodium sulfate was also present (10%, wt/vol) during dissolution of the SS in alcohol prior to reaction and during the transesterification step (8%, wt/vol). At various times during the incubation, aliquots of the reactions were removed, and their compositions were determined by HPLC. All primary alcohols examined supported transesterification. The reactions were rapid, with 90% or more of the final level of ester generally being produced within the first 30 min of incubation (Fig. 2). The appearance of ester was accompanied by a rapid and complete reduction in the glyceride and phosphoglyceride contents of the samples (Fig. 2). The exception to this observation would seem to be diglycerides, which were detected by HPLC throughout the course of the reaction (Fig. 2). We have noted multiple small peaks on HPLC traces in this region. One of these was not reduced during transesterification,

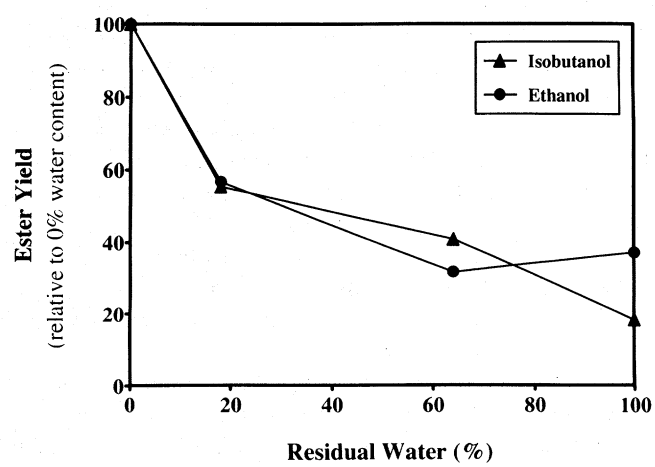

FIG. 1. Alkaline alcoholysis of soapstock (SS): effect of the water content of SS. Reactions contained 2.0 g dry-mass equivalent of partially dried SS, 1.36 mmol KOH and ethanol or isobutanol to a final volume of 12.9 mL. Reaction time: 2 h at room temperature. The yield of monohydric fatty acid ester is expressed relative to that obtained with SS that had been lyophilized to dryness ("0% residual water content").

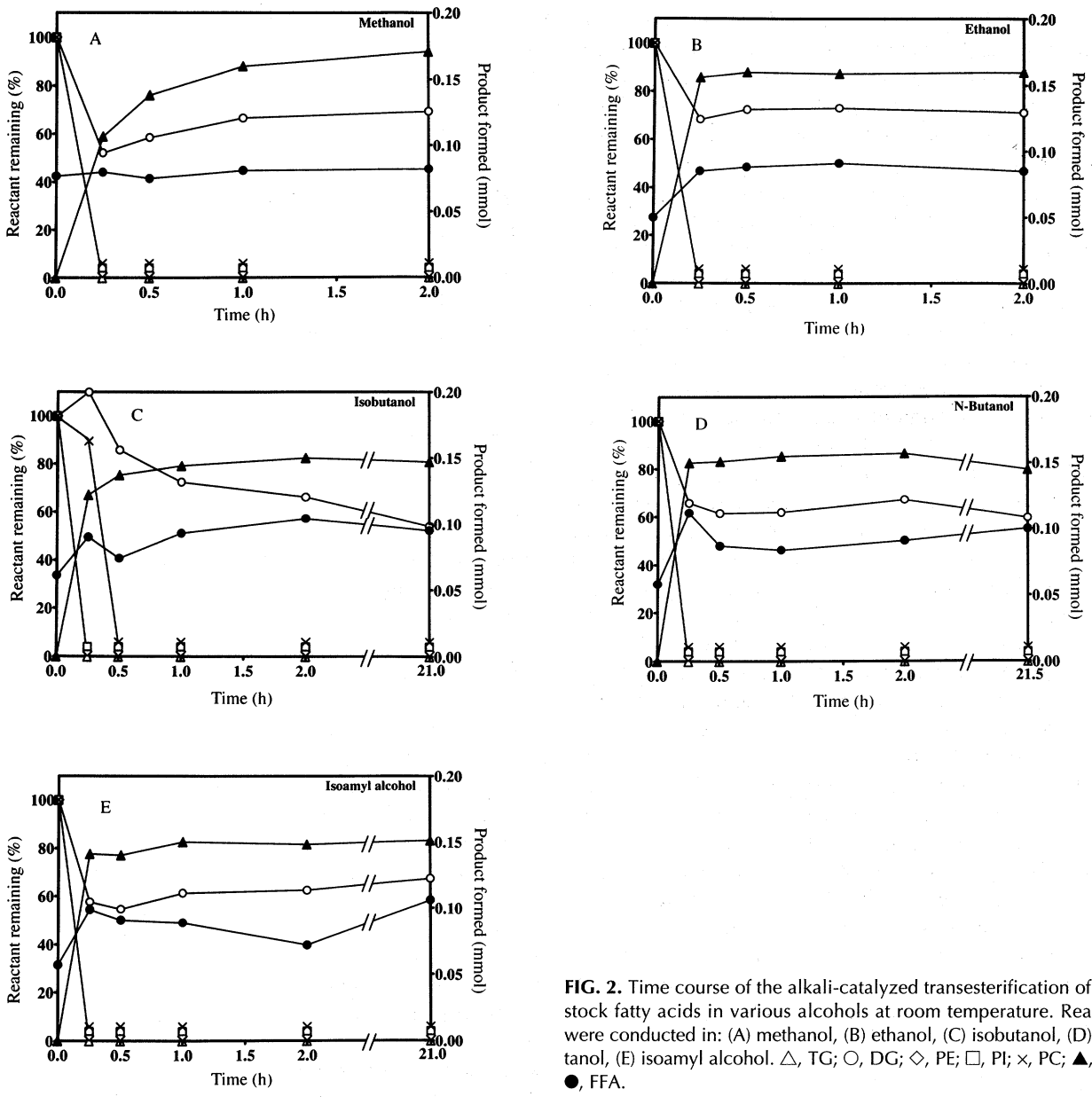

FIG. 2. Time course of the alkali-catalyzed transesterification of soapstock fatty acids in various alcohols at room temperature. Reactions were conducted in: (A) methanol, (B) ethanol, (C) isobutanol, (D) *n*-butanol, (E) isoamyl alcohol. △, TG; ○, DG; ◇, PE; □, PI; ×, PC; ▲, ester; ●, FFA.

and its area contributed to the calculated diglyceride content. Because it is unlikely that conditions that are effective at the transesterification of triglycerides, monoglycerides, and phosphoglycerides would not also esterify diglycerides, we postulate that this unreactive material is not actually diglyceride, but some other species with a chromatographic mobility similar to that of diglyceride. Due to the low content of diglycerides in SS, the amount of error introduced into the calculation of transesterification yields by the erroneous identification of this material as diglyceride is small.

Ester production continued for up to two hours, by which time all detectable lipid-linked fatty acid had been consumed. Free fatty acid levels also rose during transesterification, indicating that some lipid hydrolysis had also occurred. Others have noted that water reduces transesterification and fosters saponification (22,23). It is possible that the hydrophilic phospholipids of SS caused retention of sufficient water in the substrate to support saponification, despite extensive lyophilization. The occurrence of some hydrolysis during transesterification is not necessarily troubling, because an efficient enzymatic esterification after this reaction will convert the resulting fatty acids to esters. Little hydrolysis occurred in the methanolysis reaction, the molar ratio of transesterification to hydrolysis being 99 to 1 in this case. For the four other alcohols examined, this ratio averaged 4:1. The reaction proceeded slower in methanol than in other primary alcohols, probably due to the poor solubility of SS in this alcohol. Significant transesterification did not occur in isopropanol, a secondary alcohol, under the conditions employed here. (Isopropanol was an effective transesterifying agent when the re-

action was incubated under reflux.) When these reactions were repeated with alcohols that had not been dried with sodium sulfate, the rates and extents of ester synthesis were essentially identical to those in Figure 2 (data not shown). Therefore, drying agents were not employed in subsequent experiments.

Determination of the minimum effective amounts of alcohol and alkali. Methanol and ethanol were chosen for an investigation of the minimum amount of alcohol necessary to achieve full transesterification. Two grams of dry SS were dissolved in various amounts of these alcohols, alcoholic KOH was added to give a final concentration of at least 0.2N, and the solutions were incubated 16 h at room temperature. The yield of fatty acid esters at each alcohol concentration is shown in Figure 3. High-efficiency transesterification required a minimum 20:1 molar excess of alcohol over the lipid-linked fatty acids present in the reaction mixture. This is higher than the 2:1 to 6:1 molar excesses reported as optimal by others (11,12,23) for the transesterification of refined triglycerides. However, it is in line with reports that greater amounts of alcohol are required when the feedstock contains substantial amounts of fatty acid (24). Other unique features of the transesterifications studied here, such as the presence of unidentified materials in the heterogeneous substrate and the paste-like consistency of the reaction at low alcohol concentrations, also may have contributed to the requirement for higher amounts of alcohol.

To determine the minimum concentration of alkali necessary for full transesterification, samples of two grams of SS in either methanol or ethanol (final ratio of alcohol to esterifiable fatty acids: 20:1) were incubated for 2 h at room temperature with various concentrations of KOH, and the degrees of transesterification were determined. As shown in Figure 4, maximum transesterification required a minimum alkali concentration of approximately 0.12N. This is approximately half the alkali concentration recommended by Freedman *et al.*

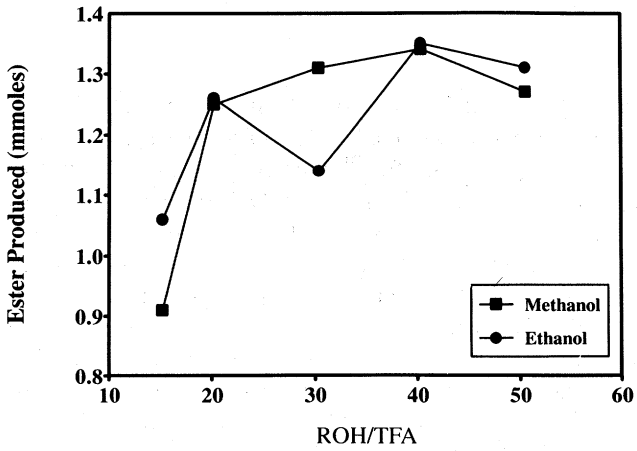

FIG. 3. Effect of the molar ratio of alcohol (ROH) to transesterifiable fatty acids (TFA) on the efficiency of soapstock transesterification in ethanol and methanol.

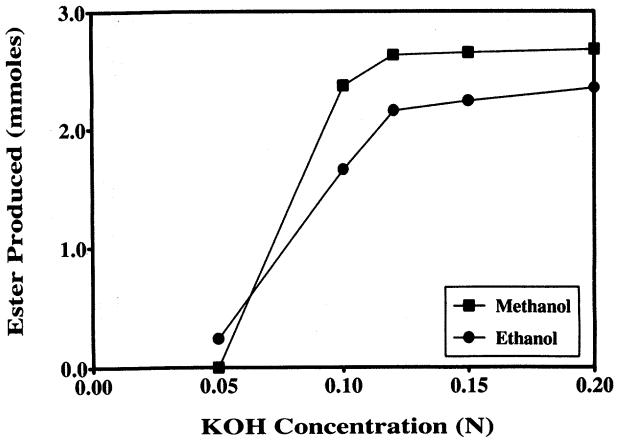

FIG. 4. Relationship between the KOH concentration and the extent of alcoholysis of soapstock in methanol and ethanol.

(12), a difference that is probably due to the lower content of transesterifiable fatty acids in SS compared to the pure triglycerides used as substrate by those investigators.

Lipase-catalyzed esterification of the free fatty acids in SS. The speed and simplicity of alkaline transesterification made it the preferred route for transesterification of the lipid-linked fatty acids in SS. However, the free fatty acids in the preparation were not esterified under these conditions. Free fatty acids can be nonenzymatically esterified under relatively strong acidic conditions (9), and a process has been described that utilizes this reaction in conjunction with a prior transesterification to produce fatty acid esters from triglyceride oils contaminated with fatty acids (24). However, the reagent and disposal costs associated with the acidification of alkaline transesterification reactions of fatty acid-rich substrates, such as SS, could be prohibitive. Therefore, the possibility of enzymatically esterifying the free fatty acids present after alkaline transesterification was investigated. Ethanol was chosen as a representative alcohol because it is a renewable, biomass-derived alcohol whose fatty acid esters serve as acceptable diesel fuels (25).

Four commercially available lipase preparations were investigated: Novo Lipozyme IM 20 and SP-435, and Amano CE and PS30. Three pH values, 13.5, 9 and 7, were studied. Only SP-435 achieved notable esterification, and only at pH 7 (Table 1). The reductions in free fatty acid levels in reactions with this enzyme were accompanied by corresponding increases in fatty acid ester levels.

Enzymes require water to display activity in nonaqueous solvents (6,26–28). The water content of the reactions in Table 1 was 0.52% (vol/vol). Because it was not known whether this water level would confer activity on all enzymes tested, the experiment was repeated at 2.2% water. Again, however, only SP-435 displayed significant activity. This activity was only one quarter of that at 0.52% water (data not shown). As a result, SP-435 was chosen for further investigation.

TABLE 1
Esterification of the Free Fatty Acids in Post-Alcoholysis Reactions by Commercial Lipase Preparations[a]

Enzyme	Amount (mg)	pH	Degree of free fatty acid esterification (%)
CE	50	13.3	1.72
CE	50	9	0.36
CE	50	7	4.18
PS30	100	13.3	0
PS30	100	9	3.46
PS30	100	7	4.63
SP-435	25	13.3	3.12
SP-435	25	9	6.48
SP-435	25	7	40.96
IM 20	100	13.3	10.56
IM 20	100	9	4.71
IM 20	100	7	9.31

[a]Alkaline alcoholysis was conducted by incubating three flasks, each containing 14 g lyophilized soapstock, 38.9 mL ethanol and 0.328 g KOH, for two hours at room temperature. The pH of two flasks was then adjusted to 7.0 and 9.0, respectively, with HCl, while the third flask remained at its original pH of 13.5. Five-mL aliquots of these solutions were dispensed to reaction tubes, and water was added to bring the water content to 0.52%. Enzyme was added, and the tubes were sealed and shaken at 42°C for 18 h. Free fatty acids were then extracted and quantitated by high-performance liquid chromatography as described in the Experimental Procedures section.

TABLE 2
pH and Water Dependence of the Esterification of Free Fatty Acids by SP-435 in Post-Alcoholysis Solutions of Soy Soapstock[a]

pH	Water content (%)	Degree of free fatty acid esterification (%)
8	0.70	7.6
7	0.70	30.4
6	0.70	42.8
7	0.54	23.3
7	1.5	17.7
7	4.0	10.0

[a]Transesterification was first conducted by incubating lyophilized soapstock (2.29 g) in ethanol (2.94 mL), containing KOH at a final concentration of 0.12 N (0.66%), for 3 h at room temperature. Concentrated HCl was then used to adjust the pH of each reaction to the desired value. Water was added to tubes at pH 7 and 8 to adjust the water content to that introduced to the pH 6 tubes by the acidification process (35 μL, 0.70%) or to higher levels as desired. SP-435 (25 mg) was added, and incubations were conducted at 42°C for 19 h. Residual free fatty acid levels were determined by high-performance liquid chromatography.

Enzyme-dependence of the esterification of SS free fatty acids by lipase SP-435. To inactivate the lipase in SP-435, samples were boiled in water for 5 min. Lipolytic assays with olive oil as the substrate indicated that this treatment reduced lipase activity by an average of 60%. In an 18-h incubation with neutralized post-ethanolysis SS, boiled enzyme was completely unable to esterify the free fatty acids present in the mixture. By contrast, under the same reaction conditions, untreated SP-435 esterified 75% of the free fatty acids. Therefore, fatty acid esterification by SP-435 in these reactions is catalyzed by the enzyme in the preparation, not the resin on which the enzyme is immobilized.

Optimal pH and water content for the esterification of free fatty acids by SP-435. Table 1 indicates that SP-435 displays greatest activity near neutral pH. To further define its pH optimum, the enzymatic esterification was conducted with post-alcoholysis samples that had been adjusted to pH 8, 7, or 6 with HCl. To avoid variations in enzyme activity due to the differing amounts of water generated by the pH adjustment process, additional water was added as needed to bring the water content of some of the pH 7 and 8 reactions to 0.70%, the water level resulting from adjustment to pH 6. It was found that the activity of the enzyme was maximum at pH 6, was reduced by 29% at pH 7, and by over 80% at pH 8 (Table 2).

To investigate the water requirements of the reaction, enzymatic esterifications were conducted at pH 7 with initial water contents of 0.54, 0.70, 1.5, and 4.0%. The reaction proceeded best at an initial water content of 0.70% and was reduced by 25% to more than 50% at the lower and higher levels examined (Table 2). The 0.70% water content corresponds to the amount of water calculated to be generated in the reactions by adjustment of the pH to 6.0. By comparison, adjustment to pH 7 was calculated to generate a water content of 0.54%. The enzyme did not exhibit as much activity in pH 7 reactions adjusted to 0.70% as it did at pH 6. Thus, the increase in activity at pH 6 is a function of both the pH change and the increased water content of the reaction.

Time course of the SP-435 esterification reaction and effect of water removal. The transesterification of 2.3 g of dry SS by KOH in ethanol was conducted in several tubes. The minimum effective alkali concentration (0.12N), the minimum effective alcohol/esterifiable fatty acid ratio (20:1), and total reaction volumes of 5 mL were employed. After a 3-h transesterification incubation, the solutions were adjusted to pH 6 and incubated at 42°C with 25 mg of SP-435 per tube. At selected times, entire reactions were removed from incubation, and their levels of free fatty acids and ethyl esters were determined. As shown in Figure 5, esterification initially proceeded

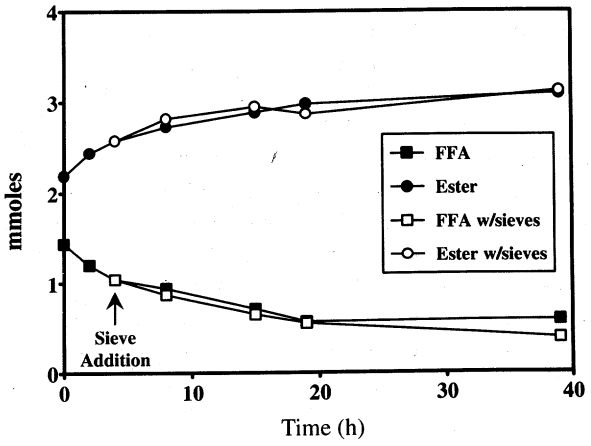

FIG. 5. Time course of SP-435-catalyzed esterification of the free fatty acids in post-ethanolysis solutions of soapstock. The addition of molecular sieves to selected samples after 4 h of incubation is indicated.

rapidly, achieving a 30% reduction in free fatty acid levels within the first four hours and a concomitant rise in ester levels. Subsequently, the reaction rate became progressively slower, with little reaction beyond 20 h of incubation.

Because excessive amounts of water destroy the activity of enzymes in organic solvents (29–32) and because the esterification reaction generates water, the addition of molecular sieves was explored as a means of increasing the degree of conversion. However, the addition of 1 g of sieves, which was calculated to be sufficient to adsorb the water generated by complete esterification of the fatty acids present in a reaction mixture, did not significantly increase the extent of reaction (Fig. 5). This may be due to the physical nature of these solutions: at the high concentration of SS employed here, two phases of approximately equal volumes were formed. One of these was a rigid plastic gum. Both the sieves and the particles of SP-435 became immobilized in this gum. Diffusional constraints under such conditions were probably quite high, reducing the effectiveness of both the sieves and the catalyst. These high viscosities were the result of the fact that, due to the attractiveness of employing minimum volumes and minimum amounts of reagents, the lowest effective volume of alcohol had been used in the reactions. Preliminary experiments, conducted at higher dilutions, have not exhibited the gumming seen here, and have achieved higher degrees of conversion (data not shown).

An overall esterification efficiency was calculated from the data generated during the time-course experiment (Fig. 5). The transesterification reaction that preceded the addition of SP-435 produced 2.43 mmole of fatty acid ethyl ester, 92% of the calculated theoretical maximum. No net ester hydrolysis occurred during transesterification: fatty acid levels after alcoholysis were equal to those at the start of the reaction. The efficiency of esterification of these free fatty acids by SP-435 over the course of a 39-h incubation was 63%, whether calculated on the basis of increases in ester content or decreases in free fatty acid level. The combined reaction resulted in the conversion of 81% of the total fatty acid content of the substrate into alkyl esters.

The two-part reaction scheme described here constitutes a potential route for the conversion of SS to higher-value material. Optimal conditions for room-temperature transesterification of SS were the use of dry substrate, a minimum molar ratio of alcohol to lipid-linked fatty acids of 20:1, and a minimum alkali concentration of approximately 0.12N. Of the enzymes tested, the best esterification of the free fatty acids present in the posttransesterification mixture occurred with lipase SP-435. At 42°C and with an enzyme dose rate of 1.1%, relative to the weight of SS, optimal esterification by this enzyme occurred at pH 6 and a water level of 0.7% (vol/vol).

Opportunities exist for additional optimization of the reaction to further increase conversion. In particular, saturated fatty acids would be solids at the temperature used here for enzymatic esterification. This could reduce their ability to serve as substrates for the enzyme. If the reaction were conducted at a sufficiently high temperature to melt these fatty acids, one would expect a greater degree of esterification to result. The technology is simple and inexpensive, and the by-products of the reaction are largely water-soluble and thus more readily disposed of than a lipid by-product. Although soy soapstock was used throughout our work, other soapstocks should readily serve as substrates. It was necessary to remove the substantial amount of water present in SS before it could be used as a substrate. However, water removal is a common and relatively inexpensive procedure in an oilseed processing operation. The first step in the reaction, alkaline transesterification, was patterned after well-defined technology, developed with triglyceride feedstocks, and achieved the alcoholysis of both the glyceride and phosphoglyceride components of the substrate. The reaction occurred rapidly and with high efficiency under ambient conditions. If needed, it should also occur readily under the higher temperatures that facilitate the separation of glycerol generated by the reaction. The addition of a second reaction, which used enzymatic catalysis at pH values near neutrality, to esterify the free fatty acids in the preparation increased overall conversion efficiency and offers an alternative to acid-catalyzed methods for this reaction.

ACKNOWLEDGMENTS

We thank Dr. Robert Moreau and Deborah Woolf for useful advice, and Dr. Lloyd Nelson for GC analysis.

REFERENCES

1. Snyder, H.E., and T.W. Kwon, *Soybean Utilization*, Van Nostrand Reinhold Co., New York, 1987, pp. 110–112.
2. Anonymous, *Soya Bluebook Plus*, Soyatech, Inc., Bar Harbor, ME, 1995, p. 262.
3. Schuch, R., and K.D. Mukherjee, Interesterification of Lipids Using an Immobilized *sn*-1,3-Specific Triacylglycerol Lipase, *J. Agric. Food Chem. 35*:1005–1008 (1987).
4. Kanasawud, P., S. Phutrakul, S. Bloomer, P. Adlercreutz, and B. Mattiasson, Triglyceride Interesterification by Lipases. 3. Alcoholysis of Pure Triglycerides, *Enz. Microb. Technol. 12*:959–965 (1992).
5. Mittelbach, M., Lipase-Catalyzed Alcoholysis of Sunflower Oil, *J. Am. Oil Chem. Soc. 67*:168–170 (1990).
6. Linko, Y.-Y., M. Lamsa, A. Huhtala, and O. Rantanen, Lipase Biocatalysis in the Production of Esters, *Ibid. 72*:1293–1299 (1995).
7. Goshray, S., and D.K. Bhattacharya, Enzymatic Preparation of Ricinoleic Acid Esters of Long-Chain Monohydric Alcohols and Properties of the Esters, *Ibid. 69*:85–88 (1992).
8. Sonntag, N.O.V., Fat Splitting, Esterification, and Interesterification, in *Bailey's Industrial Oil and Fat Products*, edited by D. Swern, Vol. 2, 4th edn., J. Wiley and Sons, New York, 1982, pp. 97–173.
9. Formo, M.W., Ester Reaction of Fatty Materials, *J. Am. Oil Chem. Soc. 31*:548–559 (1954).
10. Eisenhard, W.C., in *Fatty Acids in Industry*, edited by R.W. Johnson and E. Fritz, Marcel Dekker, Inc., New York, 1989, pp. 139–152.
11. Isigigur, A., F. Karaosmanoglu, and H.A. Aksoy, Methyl Ester from Safflower Seed Oil of Turkish Origin as a Biofuel for Diesel Engines, *App. Biochem. Biotech. 45/46*:103–112 (1994).
12. Freedman, B., E.H. Pryde, and T.L. Mounts, Variables Affect-

ing the Yields of Fatty Esters from Transesterified Vegetable Oils, *J. Am. Oil Chem. Soc. 61*:1638–1643 (1984).
13. Bradshaw, G.B., and W.C. Meuly, Preparation of Detergents, U.S. Patent 2,360,822 (1944).
14. Bloomer, S., P. Adlercreutz, and B. Mattiasson, Facile Synthesis of Fatty Acid Esters in High Yields, *Enzyme Microb. Technol. 14*:546–552 (1992).
15. Miller, C., H. Austin, L. Posorske, and J. Gonzlez, Characteristics of an Immobilized Lipase for the Commercial Synthesis of Esters, *J. Am. Oil Chem. Soc. 65*:927–931 (1988).
16. Ghoshray, S., and D.K. Bhattacharya, Utilization of Acid Oils in Making Valuable Fatty Products by Microbial Lipase Technology, *Ibid. 72*:1541–1544 (1995).
17. Vazquez Lima, R., D.L. Pyle, and J.A. Asenjo, Factors Affecting the Esterification of Lauric Acid Using an Immobilized Biocatalyst: Enzyme Characterization and Studies in a Well-Mixed Reactor, *Biotechnol. Bioeng. 46*:69–79 (1995).
18. Norris, R.A., Refining and Bleaching, in *Bailey's Industrial Oil and Fat Products*, edited by D. Swern, Vol. 2, 4th edn., J. Wiley and Sons, New York, 1982, pp. 253–314.
19. Stern, R., G. Hillion, P. Gateau, and J.C. Guibet, Preparation of Methyl and Ethyl Esters from Crude Vegetable Oils and Soapstock, in *Proceedings: World Conference on Emerging Technologies in the Fats and Oils Industry*, edited by A.R. Baldwin, American Oil Chemists' Society, Champaign, 1986, pp. 420–422.
20. Haas, M.J., D.J. Cichowicz, W. Jun, and K. Scott, The Enzymatic Hydrolysis of Triglyceride–Phospholipid Mixtures in an Organic Solvent, *J. Am. Oil Chem. Soc. 72*:519–525 (1995).
21. Haas, M.J., D. Esposito, and D.J. Cichowicz, A Software Package to Streamline the Titrimetric Determination of Lipase Activity, *Ibid. 72*:1405–1406 (1995).
22. Wright, H.J., J.B. Segur, H.V. Clark, S.K. Coburn, E.E. Langdon, and R.N. DuPuis, A Report on Ester Interchange, *Oil & Soap 21*:145–148 (1944).
23. Feuge, R.O., and A.T. Gros, Modification of Vegetable Oils. VII. Alkali-Catalyzed Interesterification of Peanut Oil with Ethanol, *J. Am. Oil Chem. Soc. 26*:97–102 (1949).
24. Sprules, F.J., and D. Price, Production of Fatty Esters, U.S. Patent 2,494,366 (1950).
25. Clark, S.J., L. Wagner, M.D. Schrock, and P.G. Piennaar, Methyl and Ethyl Soybean Esters as Renewable Fuels for Diesel Engines, *J. Am. Oil Chem. Soc. 61*:1632–1638 (1984).
26. Zaks, A., and A.M. Klibanov, The Effect of Water on Enzyme Action in Organic Media, *J. Biol. Chem. 263*:8017–8021 (1988).
27. Hirata, H., K. Higuchi, and T. Yamashina, Lipase-Catalyzed Transesterification in Organic Solvent: Effects of Water and Solvent, Thermal Stability and Some Applications, *J. Biotechnol. 14*:157–167 (1990).
28. Adlercreutz, P., On the Importance of the Support Material for Enzymatic Reactions in Organic Media, *Eur. J. Biochem. 199*:609–614 (1991).
29. Valivety, R.H., P.J. Halling, A.D. Peilow, and A.R. Macrae, Lipases from Different Sources Vary Widely in Dependence of Catalytic Activity on Water Activity, *Biochem. Biophys. Acta 1122*:143–146 (1992).
30. Valivety, R.H., P.J. Halling, and A.R. Macrae, Water as a Competitive Inhibitor of Lipase-Catalysed Esterification in Organic Media, *Biotechnol. Lett. 15*:1133–1138 (1993).
31. Svensson, I., E. Wehtje, P. Adlercreutz, and B. Mattiasson, Effects of Water Activity on Reaction Rates and Equilibrium Positions in Enzymatic Esterifications, *Biotechnol. Bioeng. 44*:549–556 (1994).
32. Haas, M.J., K. Scott, W. Jun, and G. Janssen, Enzymatic Phosphatidylcholine Hydrolysis in Organic Solvents: An Examination of Selected Commercially Available Lipases, *J. Am. Oil Chem. Soc. 71*:483–490 (1994).

[Received July 8, 1996; accepted September 3, 1996]