Enzymatic Synthesis of Acetylated Glucose Fatty Acid Esters in Organic Solvent

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Two immobilized lipases from *Candida antarctica* (SP 382) and *C. cylindraceae*, now *rugosa* (200I), catalyzed the synthesis of novel acetylated glucose fatty acid esters with glucose pentaacetate (GP) and Trisun 80 (80% oleic) vegetable oil or methyl oleate as substrates in organic solvents. The relative yield was between 6.4–52%, and the incorporation of oleic acid onto the glucose was between 31–100%. In addition, these enzymes were able to catalyze the synthesis of glucose fatty acid esters with free glucose as the sugar substrate. The highest oleic acid incorporation (100%) was obtained in benzene with SP 382 lipase and Trisun 80 as the acyl donor. With methyl oleate as the acyl donor, greater incorporation was obtained in benzene (90.5%) compared to 75% in isooctane. The 200I lipase was better in benzene/pyridine (2:1 vol/vol) (74%) and chloroform (61%) compared to benzene and isooctane. However, with free glucose and Trisun 80 as substrates, both enzymes gave acceptable levels of oleic acid incorporation (82–100%) in benzene, benzene/pyridine and pyridine. The best conditions for the ester interchange reaction reported are: lipase (10% by weight of substrate); incubation time 48 h; molar ratio of Trisun/GP 1:2; 3 mL solvent and 3% added water. These glucose esters have potential applications as emulsifiers in food, cosmetics and pharmaceutical formulations.

KEY WORDS: Acetylated glucose fatty acid esters, emulsifiers, enzymatic synthesis, ester–ester interchange, glucose fatty acid esters, glucose pentaacetate, lipases, organic solvents, transesterification.

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are now known to catalyze reverse reactions of hydrolysis in organic solvents by direct esterification with free acid, transesterification and interesterification (ester–ester interchange). This unique phenomenon is being exploited by lipid biotechnologists to modify existing fats and oils or to synthesize novel ester products with potential applications in the food, cosmetics and pharmaceutical industries. Dordick (1) and Mukherjee (2) recently reviewed several applications of lipases in the fats and oils industries. One of our current interests is on the biotechnological production of surface-active agents, such as monoglycerides, phospholipids, alkyl glycoside fatty acid esters and other carbohydrate fatty acid esters (3–5).

Acetylated monoglycerides, in addition to other emulsifiers such as lecithin, mono- and diglycerides, sorbitan esters and succinylated monoglycerides, are presently generally recognized as safe (GRAS) and are widely used in the food and pharmaceutical industries (6). Distilled acetylated monoglycerides are produced chemically by the interesterification of triglycerides, triacetin and glycerol, followed by molecular distillation, a high-energy process, to isolate a mixture of mono- and diacetylated monoglycerides, which are used as stabilizing, lubricating, plasticizing, defoaming, edible coating and emulsifying agents (7). The synthesis or use of acetylated sugar fatty acid esters for similar applications has not been reported. In fact, there are no published reports on the enzymatic synthesis of acetylated glucose fatty acid esters (AGFAE).

The major problem associated with enzymatic sugar ester synthesis is the insolubility of all the participating reactants in an acceptable solvent. Pyridine (8,9) and dimethylformamide (10) have been used as solvents in the synthesis of sugar esters catalyzed by powdered porcine pancreatic lipase and subtilisin, respectively. Janssen *et al.* (11) reported the enzymatic synthesis of sorbitol esters in aqueous media (12) and in a two-phase medium with 2-pyrrolidone as cosolvent for sorbitol, fructose and glucose esters (13). Mutua and Akoh (5) recently reported the enzymatic synthesis of alkyl glycoside fatty acid esters in organic media. Fregapane *et al.* (13) reported the solvent-free acylation of sugar acetals (isopropylidene derivatives of sugars) to produce mono- and diesters. Chopineau *et al.* (9) were the first to attempt enzymatic synthesis of sugar alcohol monoesters (not with glucose) with vegetable oils as acyl donors in nonaqueous media.

Vegetable oils are cheaper than free fatty acids and their methyl esters and may represent a good source of acyl donors during enzymatic synthesis of esters. Trisun 80 (contains approx. 80% oleic acid) and methyl oleate were chosen as the acyl donors because of the potential benefits of dietary 18:1n-9 (14). We report for the first time the enzymatic synthesis of AGFAE in organic solvent catalyzed by immobilized *Candida* lipases, SP382 and 200I.

MATERIALS AND METHODS

Materials. Methyl oleate (70% pure) and ANS salt (8-anilino-1-napthalenesulfonic acid ammonium salt, 97% pure) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Glucose pentaacetate (GP) was purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized nonspecific lipases SP 382, containing 2% water [40 batch interesterification unit (BIU) (i.e., one μmole of incorporated palmitic acid into triolein per min under standard conditions as defined by the manufacturer)/g] from *Candida antarctica*, and lipomod 200I (unspecified activity) from *C. cylindraceae (rugosa)* were from Novo Nordisk Bioindustrials Inc. (Danbury, CT) and Biocatalysts Ltd. (Wales, United Kingdom), respectively. Silica gel 60 thin-layer chromatography (TLC) plates were purchased from E. Merck (Darmstadt, Germany). Vegetable oil Trisun 80 was provided by SVO Enterprises (Eastlake, OH). All the solvents were of high-performance liquid chromatography (HPLC) grade and obtained from Fisher Scientific (Norcross, GA).

Interesterification method. Unless otherwise indicated, a typical ester–ester interchange reaction involved admixing 100 mg of GP, 152 mg Trisun 80 or methyl oleate (mole ratio, 1:2), 26 mg of immobilized SP382 or 200I lipase (i.e., 10%, w/w, of reactants) and 7.6 mg of water (i.e., 3%, w/w, of reactants) in a screw-cap test tube. To this reaction mixture was added 3 mL of solvent (e.g., isooctane or benzene), and the mixture was incubated in an orbital shaking water bath at 55°C for 48 h at 200 rpm. Reactions were carried out in duplicate. A blank control with no enzyme was incubated, and no interesterification or transesterification

activity was observed. Time course, effect of enzyme concentration, substrate ratios and solvent types on the reaction followed the same procedure with slight modifications as appropriate. Transesterification of free glucose with fatty acyl donors was also carried out by the above procedure. The water contents of the solvents were determined by coulometric Karl Fischer titration (Metrohm 684KF Coulometer; Brinkman Instruments, Inc., Westbury, NY). The solvents were stored over molecular sieve 4 Å prior to use.

Extraction and analysis. Extraction and analysis of the reaction products followed the method of Mutua and Akoh (5) for alkyl glycoside fatty acid esters. However, silica-gel 60 TLC plates were developed with a different solvent system consisting of petroleum ether/diethyl ether/acetic acid (90:10:1, vol/vol/vol). AGFAE bands (essentially mono fatty acyl esters, $R_f = 0.10$; diesters, $R_f = 0.17$) were identified by their purple-violet color after spraying with sulfuric-acid dichromate (50% H_2SO_4 solution) and charring at 110°C for 5–10 min. Unreacted Trisun 80 and methyl oleate were well separated by TLC with R_f values of 0.41 and 0.55, respectively, and gave yellow coloration after charring. For gas–liquid chromatography (GLC), the bands corresponding to the acetylated glucose or GFAE from a separate ANS-sprayed TLC plate were scraped into a test tube containing 10-μg solution (10 mg/10 mL) of heptadecanoic acid (17:0) as internal standard and methylated as previously described (5). The fatty acid methyl esters (FAME) were analyzed by GLC with an HP 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA). A DB 225 fused-silica capillary column of 30 m × 0.25 mm i.d. (J&W Scientific, Folsom, CA) was used and operated isothermally at 205°C. Injector and detector temperatures were set at 250 and 260°C, respectively, and helium was the carrier gas. The relative content of FAME as mol% was quantitated by an on-line computer with 17:0 as internal standard based on the initial amount of 18:1n-9 present. HPLC analysis of the reaction product was with a HP 1090 Win system (Hewlett-Packard, Avondale, PA), fitted with a 250 μL autoinjector and equipped with a diode array and a Sedex 45 evaporative light scattering mass detector (Richard Scientific, Novato, CA). Two gel-permeation chromatography (GPC) μSpherogel columns with 1000 and 500 Å pore-sizes, 7.7 mm i.d. × 30 cm, were connected in series (Altex, San Ramon, CA). The mobile phase was tetrahydrofuran (THF) at a flow rate of 1.5 mL/min. Twenty microliters of the reaction products dissolved in THF was injected into the HPLC after elution from a Supelco silica solid-phase extraction column. The column temperature was 40°C while the Sedex 45 detector was set at 40°C with a gain of 7. The reactants were also chromatographed to establish their retention times relative to the desired products. In addition, polystyrene molecular weight markers for organic GPC (Catalog No. 28292; Alltech, Deerfield, IL) were chromatographed, and their retention times vs. log of molecular weights were plotted to estimate the molecular weights of the products. The relative percent conversion of acyl donor to products or yield of reaction products were quantitated by an on-line computer based on total acyl donor (e.g., acetylated GFAE = 100 − relative content of Trisun 80). The composition of the major fatty acids in Trisun 80 as determined by GLC were: 16:0, 6.5; 18:0, 4.4; 18:1n-9, 80.0; and 18:2n-6, 9.1%.

RESULTS AND DISCUSSION

The relative yield of AGFAE in benzene and isooctane with trisun 80 or methyl oleate as the acyl donor, catalyzed by immobilized *C. antarctica* SP 382 lipase is detailed below. Preliminary investigation indicated that SP 382 gave higher yields than the immobilized *C. rugosa* 200I lipase. The ester yield was highest (52%) with Trisun 80 as the acyl donor and benzene as the organic solvent, followed by the reaction in isooctane (34.6%). Chloroform gave a poor yield (6.4%) of AGFAE. With methyl oleate in benzene, the yield was 35%; with methyl oleate in isooctane, the yield was 25%. Even though the yields obtained here were not high, the results do compare well with the 34% conversion of sorbitol to esters in aqueous media catalyzed by *C. rugosa* (11). In a later study, Janssen *et al.* (12) was able to obtain 80% conversion of the initial sorbitol to esters in a two-phase system with 2-pyrrolidone as a polar cosolvent. The *Candida* lipases are nonspecific with respect to fatty acid chainlength or position in the triglyceride because the product contained an identical fatty acid profile as the starting acyl donors as determined by GLC (not shown). Chopineau *et al.* (9) reported 45% yield of ribitol monoester (from the recalculation of their data) in dry pyridine, with porcine pancreatic lipase (1,3-specific) as the biocatalyst and triolein as the acyl donor. Though not yet reproduced or confirmed in our laboratory and others, Seino *et al.* (15) reported a 27.7% conversion of oleic acid to GFAE with free glucose as the substrate in a phosphate buffer of high water content. Fregapane *et al.* (13) reported 60% yield of a glucose acetal-6-monoester synthesized *via* its isopropylidene derivative in a solvent-free esterification process. However, they did not report the yield of the final glucose monoester after acid hydrolysis of the ketal group or acetone (13).

Figure 1 shows the effect of different solvents on the ability of immobilized SP 382 and 200I to catalyze the synthesis of AGFAE with methyl oleate as the acyl donor. The highest incorporation of oleic acid was obtained in benzene (90.5 mol%), followed by isooctane (75%) with SP 382 as the biocatalyst. In benzene/pyridine (2:1, vol/vol) (74% incorporation) and chloroform (61%), the 200I lipase

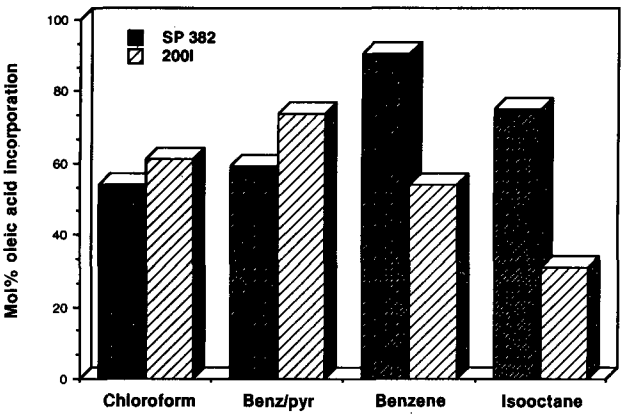

FIG. 1. Effect of different solvents on the synthesis of acetylated glucose fatty acid ester with methyl oleate as the acyl donor, catalyzed by immobilized SP 382 (Novo Nordisk Bioindustrials Inc., Danbury, CT) and 200I lipases (Biocatalysts Ltd., Wales, United Kingdom). The reaction mixtures were incubated at 55°C for 48 h at 200 rpm. Benz/pyr = benzene/pyridine.

was a better biocatalyst compared to SP 382. The reason for the unique behavior of these enzymes in different solvents is not clear at the moment. It appears that some solvents do strip out the essential water from enzymes, or that excess water from some solvents leads to hydrolysis (5). It should be noted, however, that GP was completely dissolved in chloroform, benzene/pyridine 2:1 and benzene, but not in isooctane. Lipase-catalyzed reactions in two-phase systems have been reported (11,12). Narayan and Klibanov (16) recently reported that a solvent's immiscibility with water and its apolarity (so-called log *P* values) are of no consequence on the ability of lipases and a protease to catalyze reactions. The author chose to study the other reaction parameters in isooctane (even though the yield in benzene was greater) because benzene may not be an acceptable solvent for preparing sugar esters intended for food applications. We have demonstrated the effectiveness of dual solvents like benzene/pyridine in the synthesis of alkyl glycoside fatty acid esters from polar and apolar substrates catalyzed by SP 382 lipase (5). The oleic acid incorporation in benzene in the present report (90.5%) is higher than we previously reported (19.2%), partly because the GP and methyl oleate were mutually soluble in benzene compared to methyl glucoside with four free hydroxy groups. Chloroform was not a good solvent for SP 382 lipase-catalyzed ester–ester interchange under the reaction conditions reported here. Figure 2 illustrates the effect of selected solvents on the SP 382 and 200I lipase-catalyzed synthesis (transesterification) of GFAE with free glucose as the acyl acceptor and Trisun 80 as the acyl donor. Interestingly, the levels of oleic acid incorporation were acceptable (80–90 mol%) in all the solvents tested, with benzene giving the highest incorporation (100%). No reports have been published on SP 382 and 200I lipase-catalyzed transesterification of vegetable oils with free glucose in these solvents to form GFAE. Further studies are underway in our laboratory to optimize this reaction.

Figure 3 shows the time course for SP 382 lipase-catalyzed synthesis of AGFAE with Trisun 80 as the acyl donor in isooctane. Maximum oleic acid incorporation (90%) was obtained after 48–60 h of incubation, suggesting that equilibrium may have been reached in 48 h. Beyond this reaction time, there was no improvement on the amount of oleic acid incorporated into the GP. Figure 4 illustrates the effect of varying the enzyme concentration

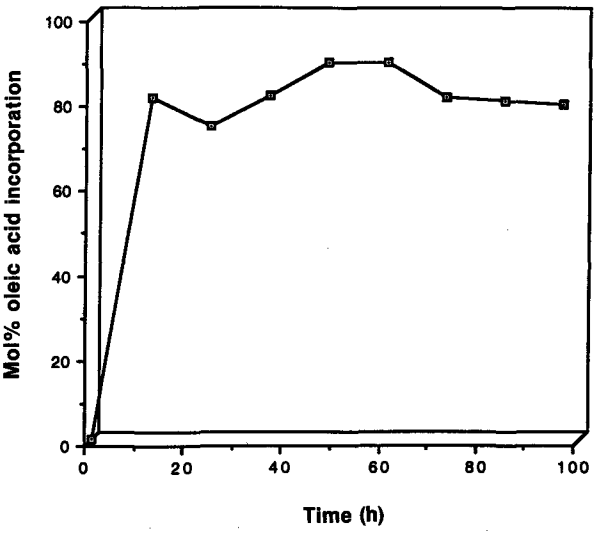

FIG. 3. Time course for immobilized SP 382 lipase-catalyzed synthesis of acetylated glucose fatty acid ester with Trisun 80 in isooctane. The reaction mixture was incubated for 96 h at 55°C with shaking at 200 rpm. A 200-μL aliquot of the reaction mixture was taken out every 12 h for analysis. See Figures 1 and 2 for company suppliers of materials.

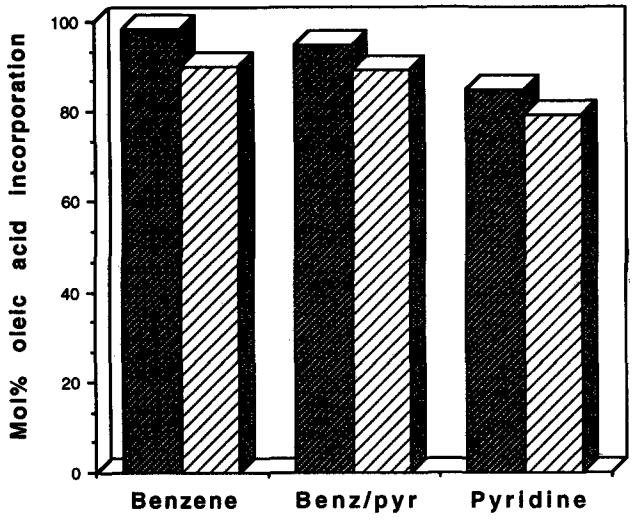

FIG. 2. Effect of solvents on the synthesis of glucose fatty acid ester with free glucose as the substrate and Trisun 80 (SVO Enterprises, Eastlake, OH) as the acyl donor. The reactions were catalyzed by immobilized SP 382 and 200I lipases. See Figure 1 legend for abbreviation, conditions and company suppliers of materials. Expt. = experiment, dark hatched bar = SP382; light hatched bar = 2001 in expt. with free glucose.

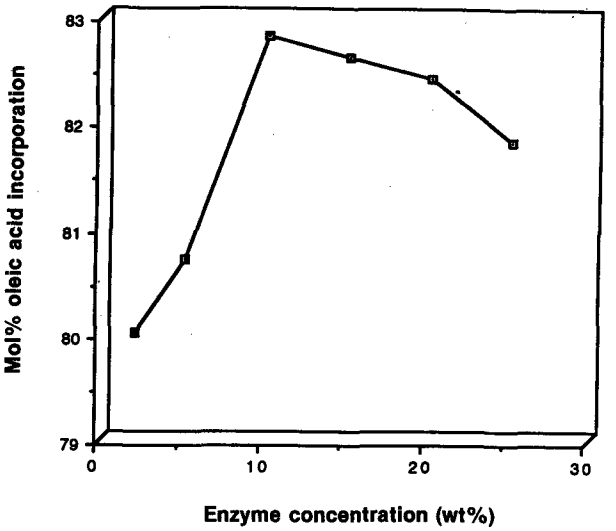

FIG. 4. Effect of varying SP 382 lipase concentration on the interesterification of glucose pentaacetate and Trisun 80 to form acetylated glucose fatty acid esters in isooctane. The reaction mixtures were incubated at 55°C for 48 h at 200 rpm. See Figures 1 and 2 for company suppliers of materials.

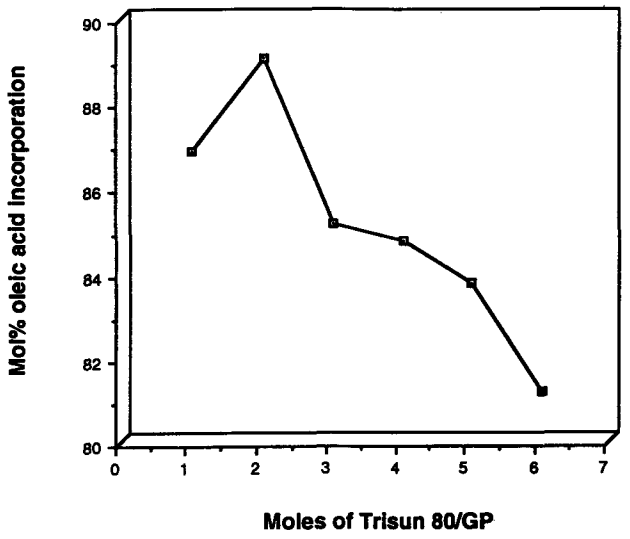

FIG. 5. Effect of varying the molar ratio of Trisun 80 to glucose pentaacetate (GP) from 1 to 6 on acetylated glucose fatty acid ester synthesis, catalyzed by SP 382 lipase in isooctane. See Figures 1 and 2 for company suppliers of materials.

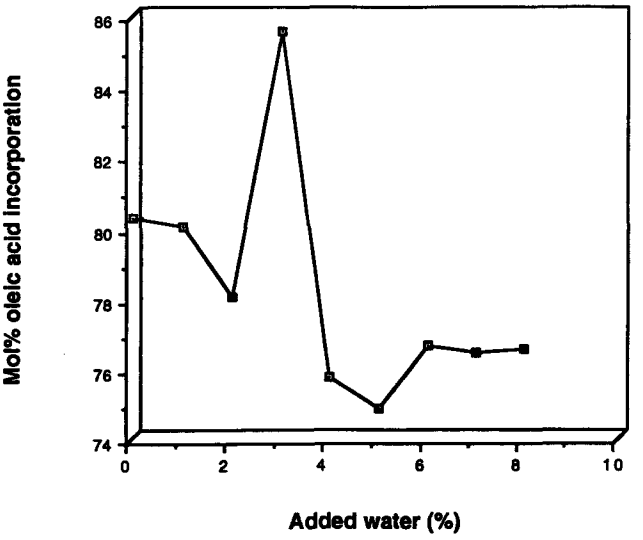

FIG. 6. Effect of additional water on the ability of immobilized SP 382 lipase to catalyze the synthesis of acetylated glucose fatty acid ester with Trisun 80 as the acyl donor in isooctane. See Figures 1 and 2 for company suppliers of materials.

(SP 382 lipase) on the interesterification of GP and Trisun 80 to produce AGFAE in isooctane. The enzyme concentration was varied from 5 to 25% by weight of reactants, and the highest amount of oleic acid was incorporated when 10% by weight of SP 382 was used. Above this level, the addition of more enzyme did not improve the amount of oleic acid inncorporated. The exact reason for this behavior remains to be determined. It may well be that 48 h was sufficient for the reaction to reach equilibrium at enzyme concentration of 10% (see Fig. 3). The manufacturer's recommended amount of this enzyme is 10% by weight of substrate. There was no further increase in the amount of AGFAE synthesized when the SP 382 concentration exceeded 10% w/w of the reactants.

Figure 5 shows the effect of varying the Trisun 80 concentration on the synthesis of AGFAE in isooctane with SP 382 lipase as the biocatalyst. Molar ratios were varied from 1:1 to 1:6, and the enzyme concentration was kept constant. The highest mol% incorporation of oleic acid (92%) was obtained when 2 moles of Trisun was reacted with 1 mole of GP. At molar ratios greater than 1:2, a sharp decline in mol% incorporation of oleic acid was obtained. The reason for this decline may be due to substrate or product inhibition of the enzyme (5). It has been suggested that a critical amount of water is necessary for enzymes to maintain their proper three-dimensional conformation and for catalysis. SP 382 lipase as supplied contained 2% water. Figure 6 shows the effect of added water (total water content) on the ability of immobilized SP 382 to catalyze the interesterification reaction with Trisun 80 as the acyl donor in isooctane. The optimum amount of water required to obtain approximately 86% oleic acid incorporation was 3% w/w, beyond which a sharp decline in incorporation was observed. The influence of water content on esterification of carbohydrates in aqueous (11) and organic solvents (5) has been reported. Recently, the influence of thermodynamic water activity, a_w (not total water content) on lipase-catalyzed esterification was reported (17,18). In fact, it was shown that *C. rugosa* lipase required high a_w (0.77) for good esterification activity, while *C. antarctica* retained activity at lower a_w (17). We have shown previously that *C. antarctica* SP 382 can catalyze transesterification reactions in organic solvents in the absence of additional water (5). The 3% additional water required in the present report is within acceptable limits for this enzyme and is probably also governed by some other factors during the reaction. Obviously, each enzyme activity is different, and factors, such as type of support, a_w, water content, drying conditions, solvent type, nature of substrates, miscibility of reactants and solvents, and other reaction conditions, are important in maximizing the synthetic ability of the lipase in question.

This report demonstrated for the first time that the syntheses of AGFAE and GFAE are possible in organic solvents, with vegetable oil and methyl oleate as acyl donors and immobilized *Candida* lipases as biocatalysts. Further studies are needed to optimize the yield of these potential emulsifiers.

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REFERENCES

1. Dordick, J.S., *Enzyme Microb. Technol. 11*:194 (1989).
2. Mukherjee, K.D., *Biocatalysis 3*:277 (1990).
3. Akoh, C.C., C. Cooper and C.V. Nwosu, *J. Am. Oil Chem. Soc. 69*:257 (1992).
4. Mutua, L.N., and C.C. Akoh, *Ibid. 70*:125 (1993).

5. Mutua, L.N., and C.C. Akoh, *Ibid. 70*:43 (1993).
6. Formo, M.W., in *Bailey's Industrial Oil and Fat Products*, edited by D. Swern, Wiley Interscience, New York, Vol. 1, 1979, pp. 255–257.
7. Eastman Food Emulsifiers, *Publication No. ZM-92B. 4*:9 (1991).
8. Therisod, M., and A.M. Klibanov, *J. Am. Chem. Soc. 108*:5638 (1986).
9. Chopineau, J., F.D. McCafferty, M. Therisod and A.M. Klibanov, *Biotech. Bioeng. 31*:208 (1988).
10. Riva, S., J. Chopineau, A.P.G. Kieboom and A.M. Klibanov, *J. Am. Chem. Soc. 110*:584 (1988).
11. Janssen, A.E.M., A.G. Lefferts and K. van't Riet, *Biotech. Lett. 12*:711 (1990).
12. Janssen, A.E.M., C. Klabers, M.C.R. Franssen and K. van't Riet, *Enzyme Microb. Technol. 13*:565 (1991).
13. Fregapane, G., D.B. Sarney and E.N. Vulfson, *Ibid. 13*:796 (1991).
14. Mattson, F., and S.M. Grundy, *J. Lipid Res. 26*:194 (1985).
15. Seino, H., T. Uchibori, T. Nishitani and S. Inamasu, *J. Am. Oil Chem. Soc. 61*:1761 (1984).
16. Narayan, V.S., and A.M. Klibanov, *Biotech. Bioeng. 41*:390 (1993).
17. Valivety, R.H., P.J. Halling, A.D. Peilow and A.R. Macrae, *Bichemica. et Biophysica. Acta. 1122*:143 (1992).
18. Valivety, R.H., P.J. Halling and A.R. Macrae, *Fed. Europ. Biochem. Soc. 301*:258 (1992).

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