Methanolysis of Seed Oils in Flowing Supercritical Carbon Dioxide

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ABSTRACT: The direct methanolysis of triglycerides in flowing supercritical carbon dioxide by an immobilized lipase is described. The reaction system consists of two syringe pumps for substrate addition and another two syringe pumps for delivering CO_2 at 24.1 MPa. Corn oil is pumped into the carbon dioxide stream at a rate of 4 μL/min, and methanol is pumped at 5 μL/min to yield fatty acid methyl esters (FAME) at >98% conversion. Direct methanolysis of soy flakes gives FAME at similar yields. This combined extraction/reaction is performed at 17.2 MPa and 50°C. The fatty acid profiles obtained for these seed oils matches those obtained by classical chemical synthesis.

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KEY WORDS: Fatty acid methyl esters, immobilized lipase, lipase, methanolysis, supercritical carbon dioxide.

The use of biocatalysts in supercritical carbon dioxide has been growing rapidly in recent years (1,2). Biocatalysts have the advantage of substate specificity under mild reaction conditions and supercritical carbon dioxide has several advantages over organic solvents (3). The solvent properties of supercritical carbon dioxide are readily modified by adjusting pressure or temperature; the diffusivity of substrates in carbon dioxide is higher than in organic solvents; carbon dioxide can easily be removed from the reaction products minimizing the need for costly downstream cleanup; when carbon dioxide is used in lieu of organic solvents, it has the additional benefit of being environmentally benigh.

Lipases in particular are amenable to syntheses in supercritical carbon dioxide. As in organic solvents (4), lipases in supercritical carbon dioxide catalyze the synthesis of esters from a variety of acids and alcohols (5–8). Lipases have been extensively applied in triglyceride technology (9–12). Lipase-catalyzed triglyceride reactions which have been carried out in supercritical carbon dioxide include interesterifications (13,14), transesterifications (15), and alcoholysis (16,17).

Fatty acid esters have a variety of uses, including antifriction agents, food preservatives, emulsifiers, and fuel alternatives. Methyl esters have been widely investigated for use as diesel fuel additives or substitutes. The large volume synthesis of fatty acid methyl esters (FAME) is accomplished by the methanolysis of fats and oils using sodium methoxide catalyst and excess methanol (18). Separation of FAME from glycerol and methanol occurs in a settling tank.

The power of supercritical carbon dioxide as a lipid solvent and reaction medium and the availability of commercial immobilized lipases present the possibility of large-scale production of FAME by means of biocatalysis. Efficient production of FAME by this approach requires a lipase with broad-substrate specificity (i.e., no positional specificity) and a flow system that can run continuously. A lipase isolated from *Candida antarctica* and immobilized on polyacrylamide has been found to be an appropriate catalyst for this purpose. A variety of ester syntheses have been catalyzed by this enzyme (19–22). We have also found that it is quantitative with respect to catalyzing methanolysis of a variety of natural triglycerides of different fatty acid compositions.

There are only a few reports of syntheses in flowing supercritical carbon dioxide (23–25). This paper describes a continuous-flow bioreactor and the conditions used for the conversion of soybean and corn oils to FAME.

MATERIALS AND METHODS

Corn oil was purchased at a local grocery. Soy flakes were prepared by the method of Galloway (26). Carbon dioxide sources were from National Welding Supply (welding grade used in the soy flake methanolysis; Bloomington, IL) and Air Products (analytical grade; Allentown, PA). High-performance liquid chromatography (HPLC)-grade methanol from Fisher Chemicals (Fairlawn, NJ) was used without further purification. Novozym 435 was obtained as a generous gift from Novo Nordisk (Danbury, CT). The immobilized enzyme is described by the manufacturer as containing 1–2% water by weight and having 7000 units/g toward propyl laurate.

Carbon dioxide was pumped with Isco, Inc. (Lincoln, NE) 100 DX syringe pumps, cooled to −10°C, and set up in a continuous-flow mode. The substrates were also pumped with Isco 100 DX pumps as shown in Figure 1.

Reactions were performed at 24.1 MPa, 50°C, at a corn oil flow of 4 μL/min. The restrictor temperature was set at 50°C to maintain a supercritical CO_2 flow of 1.0 mL/min. A steady state was established after all pressures and flows through the

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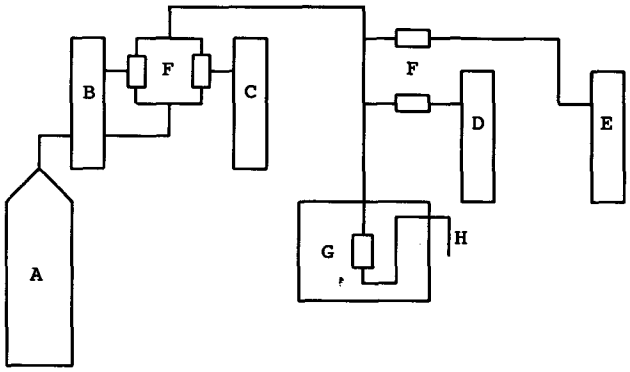

FIG. 1. Schematic of the continuous-flow system used for methanolysis of corn oil. A, CO_2 tank; B and C, CO_2 pumps; D, methanol pump; E, corn oil pump; F, check valves; G, enzyme bed in oven; H, restrictor.

system stabilized. This typically required 30 min. Products were collected in an open test tube immersed in a dry ice/isopropyl alcohol bath. Recoveries were in the range of 85–95%.

The effect of water on the transesterification was studied by flowing the CO_2 over water-saturated glass wool, inserted before the catalyst, and by adding known volumes of water to the methanol. The aqueous methanol solution was pumped at rates that maintained a methanol flow of 5 μL/min.

Methanolysis of soy flakes was performed in an apparatus represented by the schematic in Figure 2. Methanol was added with an HPLC pump (Model 100a, Beckman Instrument Inc., Fullerton, CA). The extraction vessel and the enzyme bed were connected in series as shown. The soy flakes were lyophilized prior to methanolysis in an FTS Systems Flexi-dry freeze dryer (Stone Ridge, NY). Final water content of the flakes was 2% by weight. Oil content of the flakes was 20% by weight. For the methanolysis, about 16 g flakes were placed in a stainless-steel cell (1.7 × 23 cm) and held in place with glass wool plugs. Novozym 435 (1.4 g) was placed in a separate stainless-steel vessel (0.8 × 10.2 cm) downstream from the flakes. The system was heated to 50°C and purged with CO_2 at 5.5 MPa while methanol was pumped into the system

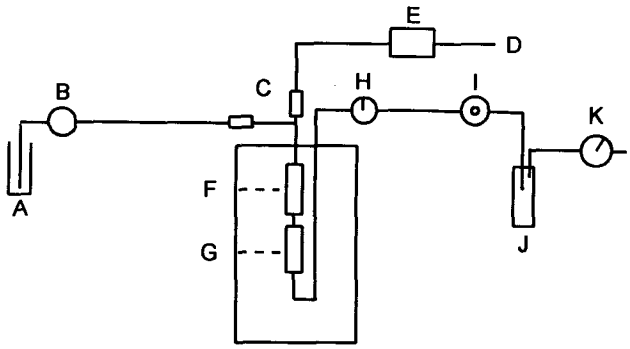

FIG. 2. Schematic of the system used for methanolysis of soy flakes. A, methanol source; B, high performance liquid chromatography pump; C, check valves, D, CO_2 source; E, gas booster; F, soy flakes and G, enzyme bed in oven; H, pressure gauge; I, micrometering valve; J, receiver; K, gas meter.

(10 μL/min). The CO_2 pressure was then increased to 17.2 MPa with a gas booster pump (Model AC-30-C; Haskel Mfg. Co., Burbank, CA). Flow was set at 8 L/min by a micrometering valve (Series 30VRMM; Autoclave Engineers, Erie, PA) as measured as expanded gas by a dry test meter.

Products were separated on a Lee Scientific Series 600 SFC/GC (Dionex, Inc., Salt Lake City, UT) with a Dionex SB-Octyl-50 column (10 m × 100 μm i.d. × 0.5 μm film). The density gradient was as follows: temperature: 100°C, 5 min, then 8°/min to 190°C; pressure: 120 atm, 5 min, then 8 atm/min to 300 atm. Samples were injected with a Valco (Valco, Inc., Houston, TX) injection loop (200 nL) held open for 1.8 s. Analytes were detected by an flame-ionization detector (FID) operating at 350°C. Conversion was determined by the ratio of the methyl ester peak area to the triglyceride peak area.

FAME were analyzed on a Hewlett-Packard 5890 Series II gas chromatograph with electronic pressure control. Instrument settings were: injector, 235°C; FID, 250°C; column head pressure, 140 kPa; carrier gas (helium) flow, 1 mL/min. FAME were separated on a Supelco (Bellefonte, PA) SP-2340 column (60 m × 0.25 mm i.d., 0.2 μm film). Chemical derivatization was accomplished by the BF_3-MeOH method (27). Glycerol was determined semiquantitatively by reversed-phase (RP) HPLC with an FID (28) and by spot test analysis (29).

RESULTS AND DISCUSSION

Methanolysis of corn oil. A schematic of the flow system used to produce FAME from corn oil is shown in Figure 1. Conversions were performed at 24.1 MPa and 50°C, conditions consistent with the maintenance of enzyme activity. This corresponds to a CO_2 density of 0.83 g/mL. Corn oil was pumped at a flow of 4 μL/min when CO_2 flow was at 1 mL/min. At higher flow of corn oil or lower pressures, oil precipitates onto the exterior walls of the reaction cell; therefore, reactions were performed at the above conditions.

In a typical run, 425 mg corn oil was pumped over 500 mg Novozym 435 to yield 365 mg product. The product was usually >95% FAME, with the balance of products being mono-, di-, and triglycerides. Upon standing at room temperature, colorless crystals precipitated out of the FAME solution. RP-HPLC determined that these crystals were monoglycerides.

Characterization of methyl ester products. The fatty acid composition of corn and soybean oils as determined by chemical derivatization (BF_3/methanol) and by methanolysis with Novozym 435 is presented in Table 1. The FAME profiles are identical, regardless of the derivatization method used, indicating that the methanolysis as catalyzed by Novozym 435 was nonspecific and complete.

Isolation of glycerol. It was anticipated that the low solubility of glycerol in supercritical carbon dioxide might inhibit methanolysis, due to precipitation of glycerol onto the enzyme bed. However, only a small amount of glycerol was ever found on the catalyst. Analysis by RP-HPLC showed

TABLE 1
Comparison of Fatty Acid Methyl Ester Distribution Obtained by Enzymatic Conversion in Supercritical Carbon Dioxide vs. Chemical Transesterification (n = 2)

Fatty acid	Corn oil		Soybean oil	
	BF_3 assay[a]	Enzymatic assay	BF_3 assay	Enzymatic assay
16:0	11.0	11.6	10.4	10.3
18:0	2.1	2.3	3.9	3.9
18:1	25.9	25.5	22.9	22.9
18:2	57.7	57.4	53.9	53.9
18:3	0.9	0.9	7.1	7.2

[a]See Reference 33.

that less than 10% of the excess glycerol was found on the enzyme bed. The remainder was found in the product. Following a reaction in which 1.02 g corn oil was transesterified, the product was dissolved in hexane (10 mL), and this layer was washed with methanol (10 mL). Removal of the solvents yielded 772 mg FAME and 78 mg glycerol, respectively. This is a recovery of 82% (total mass based on 1.04 g corn oil), and the glycerol mass is ≈10% that of the FAME, which is the mass expected based on the respective formula weights for corn oil (≈861).

The isolation of glycerol in the receiver suggests that glycerol solubility in a CO_2/methanol mixture is high enough to prevent the glycerol from poisoning the catalyst. It also makes the biocatalytic route to FAME and glycerol much like the chemical route.

Effect of methanol flow. The effect of changing methanol flow is shown in Figure 3. When corn oil flow was held at 4 μL/min, the production of FAME was greatest at a methanol flow of 5 μL/min. At lower flows, methanol is limiting, and at higher flows, methanol inhibition occurs. This 4:5 flow ratio is a stoichiometry of 25 methanol/triglyceride.

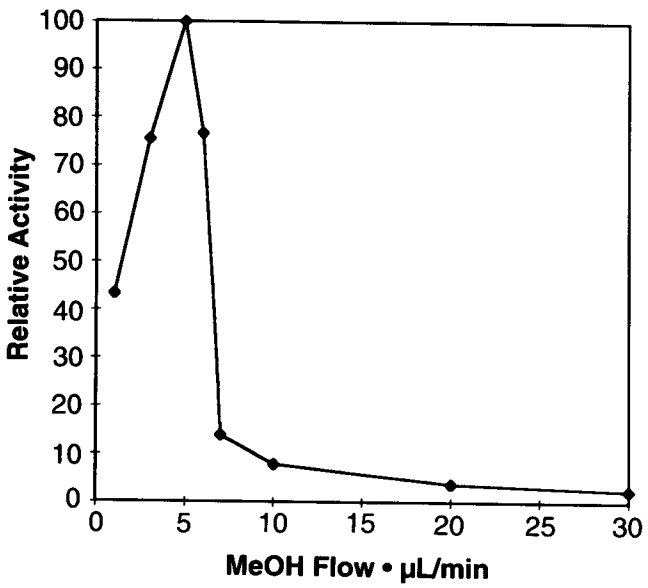

FIG. 3. The effect of methanol flow on methanolysis of corn oil. Conditions are described in the text.

Effect of water content. The effect of water on enzymes in hydrophobic media has received much attention. A small amount of water is required to maintain enzyme activity (30), but excess water can inhibit reactions by promoting hydrolysis (21,31). The effect of increasing water content on the methanolysis of corn oil was inhibitory. With the CO_2 stream saturated with water, enzymatic activity was reduced about 90%. This may be caused by a drop in pH due to the formation of carbonic acid (16). No hydrolysis products were found, i.e., fatty acids, and none are formed when water is added without methanol. This indicates that hydrolysis does not compete with methanolysis under these conditions. The effect of smaller amounts of added water on methanolysis of corn oil in supercritical carbon dioxide is as follows: 0, 0.05, 0.1, 0.2, 0.3 volume percentage of water in CO_2 to 100, 99, 81, 56, 18 relative activity, respectively. Inhibition becomes pronounced at 0.1% (vol/vol CO_2) water. The immobilized Novozym 435 was found to be active without added water. It is possible that enough water was present in the CO_2, methanol, and corn oil to keep the enzyme hydrated.

Methanolysis of soy flakes. Single-step production of FAME from soy flakes was found to work best at lower pressures. At 24.1 MPa, conversions were about 67%, while at 17.2 MPa, conversions were >98%. The solubility of soybean oil at 24.1 MPa is about three times as high as it is at 17.2 MPa. This suggests that, at the higher pressure, oil is extracted from the flakes faster than the enzyme can catalyze the methanolysis. The higher pressure may also reduce enzymatic activity.

Complete extraction/methanolysis of 15.32 g of soy flakes at 17.2 MPa took approximately 9 h. The products were collected and dissolved in hexane. The hexane layer was extracted with methanol to recover 220 mg glycerol. From the hexane layer were recovered 3.09 g FAME, which also contained a small amount of monoglycerides. Supercritical fluid chromatography analysis of the hexane-soluble mixture showed that the monoglyceride content was <1%. Diglycerides and unreacted soybean oil found in the hexane layer were also of this magnitude.

The system described here for using flowing CO_2 and an immobilized biocatalyst is versatile, and Novozym 435 was found to be an effective catalyst in such a system. The dual substrate pumps allow the addition of any liquid substrate or substrate dissolved in a carrier, and precise control over flow rates allows for control of stoichiometry. However, the low solubility of triglycerides at pressures amenable to biocatalysts limits the usefulness of this application. The use of a solid-phase acid ctalyst (32) that can withstand higher pressures could make methanolysis of triglycerides in supercritical carbon dioxide more practicable. Other syntheses in supercritical carbon dioxide with the described system are underway.

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REFERENCES

1. Aaltonen, O., and R. Markku, Biocatalysis in Supercritical CO_2, *Chemtech. 21*:240–248 (1991).
2. Nakamura, K., Biochemical Reactions in Supercritical Fluids, *Trends Biotech. 8*:288–292 (1990).
3. Clifford, A.A., Reactions in Supercritical Fluids, in *Supercritical Fluids, Fundamentals for Application,* edited by E. Kiran, and J.M.H. Levelt Sengers, Kluwer Academic Publishers, Dordrecht, 1994, pp. 449–479.
4. Klibanov, A.M., Enzymatic Catalysis in Anhydrous Organic Solvents, *Trends Biochem. Sci. 14*:141–144 (1989).
5. Marty, A., W. Chulalaksananukul, J.S. Conderet, R.M. Willemot, and G. Durand, Comparison of Lipase-Catalyzed Esterification in Supercritical Carbon Dioxide and in *n*-Hexane, *Biotechnol. Lett. 12*:11–16 (1990).
6. Dumont, T., D. Barth, C. Corbier, G. Branlant, and M. Perrut, Enzymatic Reaction Kinetic: Comparison in an Organic Solvent and in Supercritical Carbon Dioxide, *Biotechnol. Bioeng. 39*:329–333 (1992).
7. Goldberg, M., F. Parvaresh, D. Thomas, and M.-D. Legoy, Enzymic Ester Synthesis with Continuous Measurement of Water Activity, *Biochim. Biophys. Acta 957*:359–362 (1988).
8. Cernia, E., C. Palocci, F. Gasparrini, D. Misitu, and N. Fagnano, Enantioselectivity and Reactivity of Immobilized Lipase in Supercritical Carbon Dioxide, *J. Mol. Catalysis 89*:L11–L18.
9. Rattray, J.B.M., Biotechnology and the Fats and Oils Industry—An Overview, *J. Am. Oil Chem. Soc. 61*:1701–1712 (1984).
10. Quinlan, P., and S. Moore, Modification of Triglycerides by Lipases: Process Technology and Its Application to the Production of Nutritionally Improved Fats, *INFORM 4*:580–585 (1993).
11. Shishikura, A., K. Fujimoto, T. Kaneda, K. Arai, and S. Saito, Modification of Butter Oil by Extraction with Supercritical Carbon Dioxide, *Agric. Biol. Chem. 50*:1209–1215 (1986).
12. Gioielli, L.A., R.N.M. Pitombo, M. Vitolo, R. Baruffaldi, M.N. Oliveira, and M.S. Augusto, Acidolysis of Babassu Fat Catalyzed by Immobilized Lipase, *J. Am. Oil Chem. Soc. 71*:579–581 (1994).
13. Chi, Y.M., K. Nakamura, and T. Yano, Enzymatic Interesterification in Supercritical Carbon Dioxide, *Agric. Biol. Chem. 52*:1541–1550 (1988).
14. Shishikura, A., K. Fujimoto, T. Suzuki, and K. Arai, Improved Lipase-Catalyzed Incorporation of Long-Chain Fatty Acids into Medium-Chain Triglycerides Assisted by Supercritical Carbon Dioxide Extraction, *J. Am. Oil Chem. Soc. 71*:961–967 (1994).
15. Pasta, P., G. Mazzola, G. Carrea, and G. Riva, Subtilisin-Catalyzed Transesterification in Supercritical Carbon Dioxide, *Biotechnol. Lett. 2*:643–648 (1989).
16. Berg, B.E., E.M. Hansen, S. Gjørven, and T. Greibokk, On-Line Enzymatic Reaction, Extraction, and Chromatography of Fatty Acids and Triglycerides with Supercritical Carbon Dioxide, *J. High Res. Chromatogr. 16*:358–363 (1993).
17. Gunnlaugsdottir, H., and B. Sivik, Lipase-Catalyzed Alcoholysis of Cod Liver Oil in Supercritical Cqarbon Dioxide, *J. A Oil Chem. Soc. 72*:399–405 (1995).
18. Farris, R.D., Methyl Esters in the Fatty Acid Industry, *Ib 56*:770–773 (1979).
19. Johnson, C.R., and H. Sakaguchi, Enantioselective Transest fications Using Immobilized, Recombinant *Candida antarct* Lipase B: Resolution of 2-iodo-2-Cycloalken-1-ols, *Syn 10*:813–816 (1992).
20. Claon, P.A., and C.C. Akoh, Enzymatic Synthesis of Gera Acetate in *n*-Hexane with *Candida antarctica* Lipases, *J. A Oil Chem. Soc. 71*:575–579 (1994).
21. Huang, K-h., and C.C. Akoh, Lipase-Catalyzed Incorporation n-3 Polyunsaturated Fatty Acids into Vegetable Oils, *Ib 71*:1277–1280 (1994).
22. Scheckermann, C., A. Schlotterbeck, M. Schmidt, V. Wray, S. Lang, Enzymatic Monoacylation of Fructose by Two Pro dures, *Enz. Microbial. Technol. 17*:157–162 (1995).
23. van Eijs, A.M.M., J.P.L. de Jong, H.J. Doddema, and D.R. L deboom, Enzymatic Transesterification in Supercritical Cart Dioxide, *Proceedings of 1st International Symposium Superc ical Fluids,* Nice, INP, Lorraine, 1988, pp. 933–942.
24. Miller, D.A., H.W. Blanch, and J.M. Prausnitz, Enzyme-C alyzed Interesterification of Triglycerides in Supercritical C bon Dioxide, *Ind. Eng. Chem. Res. 30*:939–946 (1991).
25. Marty, A., D. Combes, and J.-S. Condoret, Continuous Re tion-Separation Process for Enzymatic Esterification in Sup critical Carbon Dioxide, *Biotechnol. Bioeng. 43*:497–5 (1994).
26. Galloway, J.P., Cleaning, Cracking, Dehulling, Decorticati and Flaking of Oil-Bearing Materials, *J. Am. Oil Chem. S 53*:271–274 (1976).
27. Metcalf, L.D., and A.A. Schmitz, The Rapid Preparation Fatty Acid Methyl Esters for Gas Chromatographic Analy *Anal. Chem. 33*:363–364 (1961).
28. Neff, W.E., E. Selke, T.L. Mounts, W. Rinsch, E.N. Frank and M.A.M. Zeitoun, Effect of Triacylglycerol Composition Structures on Oxidative Stability of Oils from Selected Soyb Germplasm, *J. Am. Oil Chem. Soc. 69*:111–118 (1992).
29. Feigl, F., *Spot Tests in Organic Analysis,* Elsevier Publishi Co., New York, 1966, p. 130.
30. Dordick, J.S., Enzymatic Catalysis in Monophasic Organic S vents, *Enz. Microbial. Technol. 11*:194–211 (1989).
31. Steytler, D.C., P.S. Moulson, and J. Reynolds, Biotransforn tions in Near-Critical Carbon Dioxide, *Ibid. 13*:221–226 (199
32. Vieville, C., Z. Moulougui, and A. Gaset, Esterification of Ol Acid by Methanol Catalyzed by *p*-Toluenesulfonic Acid and Cation-Exchange Resins K2411 and K1481 in Supercritical C bon Dioxide, *Ind. Eng. Chem. Res. 32*:2065–2068 (1993).
33. *Official Methods and Recommended Practices of the Americ Oil Chemists' Society,* American Oil Chemists' Society, Cha paign, 1969, Method Ce 2–66.

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