Oat (*Avena sativa*) Caryopses as a Natural Lipase Bioreactor[1]

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The caryopses of oats, when moistened and immersed in oils, constitute a natural lipase bioreactor. Hydrolysis was monitored by titration of the free fatty acids in the oil phase and by thin-layer chromatography. The optimum amount of additional water was about 20% of the weight of the caryopses, and the optimum temperature was about 40°C. The reaction was accelerated by gentle agitation, by reducing the viscosity of the oil phase by the addition of nonpolar solvents, and by increasing the amount of lipase on the caryopses. The reaction was inhibited by the accumulation of glycerol in the interior of the caryopses and free fatty acids in the oil phase. The lipase hydrolzyed all three positions of glycerol and there was little accumulation of mono- or diglyceride in the lipid phase. The time necessary to obtain 90% hydrolysis varied for a few days to several weeks. Greater degrees of hydrolysis could be obtained by replacing the caryopses when they became inhibited or by diluting the oil phase with hexane. The glycerol that was released could be recovered by extracting the caryopses with water. The moist oat bioreactor also was capable of catalyzing transesterification and interesterification reactions.

KEY WORDS: Ester synthesis, fat hydrolysis, lipase.

Most industrial hydrolysis of fats and oils is achieved with the Colgate-Emery process, which splits esters with high-pressure steam (1,2). Numerous proposals have been made to replace this process with gentler and less energy-intensive enzymatic processes (3–10), but none of these has gained wide acceptance.

Oats long have been known to be rich in lipase, and oat processing usually begins with a steam treatment of the caryopses to inactivate the lipase immediately after dehulling (11). Martin and Peers (12) demonstrated that much of this lipase is on the surface of oat caryopses, and the lipase may be removed by wet scrubbing of the surface.

This paper reports the use of moistened oat caryopses as a lipase bioreactor for the hydrolysis of vegetable oils. Various factors affecting the hydrolysis were investigated. Oat caryopses also were shown to be capable of catalyzing certain ester synthesis and transesterification reactions.

METHODS

Oats were obtained from the Department of Agronomy, Iowa State University, Ames, Iowa. They were stored at 4.4°C and 40% relative humidity after harvest. The oats were dehulled with an experimental-scale dehuller (Quaker Oats Co., Chicago, IL). Oat lipase concentrates were obtained by tumbling 100 g of caryopses with 250 mL of water and 200 1.8-cm porcelain balls at 60 rpm in a 20-cm ball mill (U.S. Stoneware, Akron, OH) for 15 min. The aqueous extract was centrifuged at 990 × *g* for 10 min, and the supernatant was freeze dried, yielding 2.4 g of solids. Lipases from species other than oats, fatty acids, and alcohols were obtained from Sigma (St. Louis, MO). The oils were refined, commercial products. Oat selections were screened for lipase activity by using tributyrin agar (13). The agar was prepared by mixing, at 100°C, 0.086 mL of tributyrin, 2.15 mL of a 10% aqueous solution of Tween 80, 0.43 mL of a 1% aqueous solution of merthiolate and 5.33 mL of water. The resulting emulsion was mixed with 35 mL of nutrient agar at 80–90°C and poured into Petri plates. Duplicate caryopses were pressed into the agar and incubated for 17 hr at 37°C. Lipase activity was judged by comparing the size of clear zones around the caryopses with those from a standard oat variety.

The reaction system consisted of caryopses wetted with 0.05 to 0.3 mL/g of water and immersed in 1 to 1.55 g oil (or oil diluted with solvent)/g caryopses in temperature-controlled water baths or incubators. The reaction mixtures sometimes were stirred gently with magnetic stirrers or by shaking. Phosphate buffer (0.24 molar, pH 7.5) or a "universal" buffer (14) was used to adjust the pH of the caryopses. The universal buffer was 28.57 mmolar in boric, citric, diethylbarbituric, and phosphoric acids. The desired pH was obtained by adjustment with 1 N hydrochloric acid or sodium hydroxide. When external lipases were added to the caryopses, 950 to 7,500 units of commercial lipase or 0.4–1.1 g of freeze-dried oat lipase were either dispersed in the water (0.2 mL/g) used to wet the caryopses or added directly to the reaction mixtures after the oil had been added. To test for lipase inhibition by free fatty acids in the oil phase, fresh, moistened caryopses were immersed in soybean oils that were 55 or 78% hydrolyzed by previous exposure to moist caryopses. In experiments designed to achieve complete hydrolysis with added lipase, 1504 units of *Candida rugosa* lipase/g caryopses were added to the oil phase. To study incorporation of free fatty acids into soybean oil by moist oat caryopses, 1.55 g of mixtures of fatty acids and soybean oil that were equimolar on a fatty acid basis were used/g caryopses. The caryopses were moistened with 0.1 or 0.3 mL of water/g caryopses. For a survey of the ability of oil-soluble alcohols to react, moist caryopses (0.2 mL/g caryopses) were immersed in a mixture of 1.55 g soybean oil and 1 mL of alcohol/g caryopses. To test the effect of excess alcohol, 1.55 g of mixtures of soybean oil and alcohol (1:3 by weight)/g caryopses were used. Water-soluble alcohols such as glycerol were tested by dissolving 92 or 184 mg in the 0.1 or 0.3 mL of water used to moisten the caryopses. The caryopses were immersed in 1.7 g oleic acid/g caryopses. To test the ester-forming ability of sugars or sorbitol, the 0.2 mL water that was used to moisten each gram of caryopses was saturated with the sugar

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or sorbitol before the caryopses were immersed in soybean oil (1.55 g/g caryopses).

The extent of reaction was monitored by titration, and thin-layer chromatography was used to note the appearance, disappearance and relative amounts of various components. For titration, 0.2 g of the oil phase was dissolved in 15 mL of diethyl ether-methanol, 2:1 v/v, and a 5-mL aliquot of the solution was diluted with 50 mL of methanol and titrated to pH 9 with 0.01 N sodium hydroxide solution. Thin-layer plates of silica gel G, 0.25 mm thick, were developed in hexane/diethyl ether/acetic acid, 84:15:1, v/v/v, and spots were detected under ultraviolet light after the plates had been sprayed with 0.2% 2′,7′-dichlorofluorescein in ethanol. To determine the fatty acid composition of triglycerides, reaction mixtures were streaked on thin-layer plates and the triglyceride band was extracted, transesterified, and analyzed by gas chromatography (15).

To study the rate of extraction of glycerol from caryopses with water, caryopses that had been used to achieve 26.5% hydrolysis of soybean oil were packed into a 2.5-cm column, water in sufficient quantity to cover the oats was circulated through the caryopses at 60 mL/min, and the glycerol content of the water was determined by evaporating the water in a rotary evaporator and analysis by the gas chromatographic method of Molever (16).

RESULTS AND DISCUSSION

Effect of water on hydrolysis rate. Figure 1 shows the effect of the amount of water added to the caryopses on the extent of hydrolysis for the first five days and after 13 days of reaction. There was some hydrolysis by the oats without added water, but for the first 5 days, the reaction was fastest with 0.05 mL water/g caryopses. Oats containing 0.3 mL water/g caryopses (data not shown) gave less hydrolysis than those with 0.2 mL/g. Initially excess water may form a surface coating on the caryopses and serves as a mechanical barrier that prevents effective contact between the oil and lipase. After 13 days, the greatest hydrolysis was observed in the mixture containing 0.2 mL water/g caryopses probably because water was used up in the hydrolysis reaction and was bound by the glycerol and free fatty acids that accumulate during the reaction. Thus, water became a limiting factor in the reaction mixtures containing lesser amounts. The reaction was fairly slow. At 5 days, the maximum hydrolysis was about 25% and, after 13 days, about 60%.

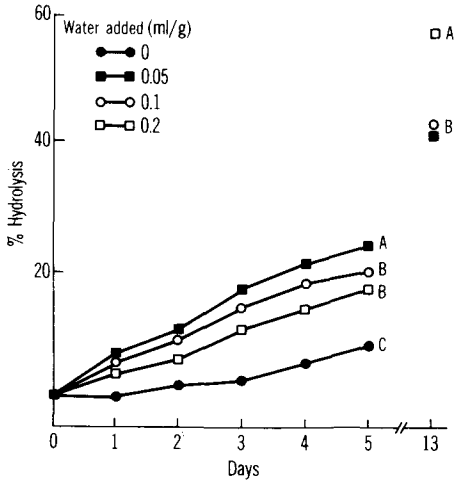

FIG. 1. The effect of the amount of water on the hydrolysis of soybean oil by oat caryopses in an unagitated system. Reaction temperature was 36°C. Treatments were compared statistically at 5 and 13 days, and those with the same letter were not significantly different at $p<0.05$.

Replicate reaction mixtures gave close agreement with standard deviations of about 1% hydrolysis. Replicate titrations of the same reaction mixture had standard deviations of about 0.42% hydrolysis.

Initial hydrolysis rates were about 67% greater when the caryopses were allowed to soak up the water (0.2 mL/g caryopses) before the oil was added than when the water was added after the oil. Soaking the caryopses in water gave hydrolysis rates that were 40% better than those obtained by adding the water as an emulsion in the oil. About 2 hr of soaking were required for the oat caryopses to absorb 0.2 mL of water/g. Longer equilibration times before the addition of the substrate oil had no appreciable effect on the hydrolysis rate.

Thin-layer chromatograms of the reaction mixtures showed that free fatty acids were the major products with little accumulation of mono- and diglycerides.

Although oats normally would mold quickly at the moisture contents used in some of these studies, no tendency for microbial growth of deterioration of the oats was observed even after several months as long as the oats were immersed in oil. If the oats were not covered with oil, mold was observed.

The effect of temperature on hydrolysis rates. Comparison of hydrolysis rates at 30, 40, 50, and 60°C showed that the rate at 40°C was about 25% greater than at 30°C. The enzyme had an optimum temperature between 40 and 50°C. At 60°C the enzyme had very little activity. This temperature sensitivity would be a disadvantage in the hydrolysis of fats with great amounts of saturated long-chain fatty acids because the fat phase would not be liquid at temperatures the enzyme could tolerate.

The effect of agitation and dilution of the substrate with solvents. Agitation increased the rate of hydrolysis, and the effect increased with extent of hydrolysis. For example, a stirred mixture gave 1.3 times as much product as an unstirred one after one day, 1.5 times as much after three days, and 1.9 times as much after five days. This suggests that the rate of unstirred reactions was limited by diffusion, especially as fatty acids began to accumulate in the medium.

The rate-limiting effect of diffusion also was demonstrated by decreasing the viscosity of the oil phase with hexane as shown in Figure 2. Dilution of the oil in an unstirred reaction with up to 50% hexane increased the release of free fatty acids significantly. For the period from 2 to 14 hr, there was relatively little increase in the total amount of fatty acids released by diluting the oil phase with more than 20% hexane. Dilution to give more than 50% hexane in the oil phase slowed the release of free fatty acids. Presumably this is because the substrate concentration becomes limiting in the more dilute solutions. However, since the total amount of free fatty acid released was not affected very much by dilution during the initial stages of the reaction, and because the more diluted samples contained less oil to be hydrolyzed, the per-

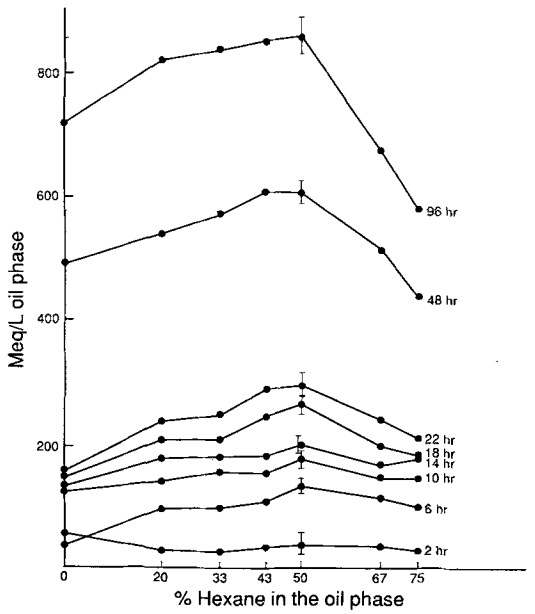

FIG. 2. The effect of dilution of the soybean oil substrate with hexane on the release of free fatty acids at various times. Conditions: 0.2 mL water/g caryopses, no agitation, 1.0 mL oil-hexane phase/g caryopses. Each point is the mean of four observations, and the bar at 50% hexane is the least significant difference for the means at p<.05. %Hydrolysis = [(meq/L)/3156][100/(100–%hexane)]100.

centage of hydrolysis of the diluted oils increased in proportion to their dilution. Thus, at 96 hr and at hexane percentages of 0, 50, 67, and 76, the hydrolysis was 22, 52, 61, and 70%, respectively. Undiluted reactions plateaued at about 60% hydrolysis at 13 days. The higher percentages of hydrolysis obtained for the most diluted samples suggest that they were not as inhibited by the accumulation of products as were the undiluted samples.

The value of the various solvents that were tested for diluting the substrate oil was not the same (Table 1). The advantage of dilution seemed to increase with decreasing chain length of hydrocarbons as would be expected if lowering of viscosity was the mechanism causing this effect. More polar solvents were less effective than hydrocarbons in accelerating the rate of hydrolysis, possibly because they bound water or partly denatured the lipase. Martin and Peers (12) reported similar effects with solvents used to defat oats before testing them for lipase activity.

Effect of buffers. A universal buffer containing borate, phosphate, citrate, and diethylbarbituric acid and adjusted to pHs from 1 to 13 was substituted for the water (0.2 mL/g) used to moisten the caryopses in an attempt to control the pH. Caryopses moistened with universal buffer adjusted to pH 11 gave the greatest amount of hydrolysis for the buffered samples, but caryopses moistened with water alone gave 37% more hydrolysis at 48 hr than the best of the buffered oats. As the concentration of universal buffer used to wet the caryopses was decreased, the rate of hydrolysis approached that obtained with water. Phosphate buffer (0.24 molar) at pH 7.5, the reported pH optimum of oat lipase (17), when substituted for water at 0.2 mL/g caryopses gave only 68% of the amount of hydrolysis obtained with water.

TABLE 1

The Extent of Hydrolysis After 1 Day of Soybean Oil Diluted 1:1, v:v with Various Solvents[a]

Solvent	% Hydrolysis
Hexane	21.5^{AB}
Heptane	22.5^{A}
Octane	20.7^{B}
Iso-octane	20.5^{B}
Nonane	20.5^{B}
Cyclohexane	17.5^{C}
Benzene	5.5^{F}
Acetone	0^{G}
Chloroform	0.6^{G}
Diethyl ether	13.7^{D}
Control (without solvent)	8.8^{E}

[a]Conditions: 0.2 mL water/g caryopses, 1 mL of oil phase/g caryopses, no agitation, 25°C. For samples with solvent, meq/L oil phase = % hydrolysis × 15.78. For the control, the factor is 31.56. Values with the same letter are not significantly different at $p<0.05$.

Seemingly inhibition of lipase activity by the water-binding ability of the buffer salts outweighs any gain from pH adjustment.

Effect of added lipase. Freeze-dried oat lipase added at 37 mg/g caryopses, accelerated the initial rate of hydrolysis of oat bioreactors 1.8-fold, and 110 mg/g caryopses gave a 3.5-fold increase. This showed that moist oat bioreactors were rate-limited by enzyme concentration as well as by diffusion. As hydrolysis proceeded and product inhibition occurred, the reactions all attained a similar degree of hydrolysis. The method of adding the lipase was important. When crude oat lipase was added to the water used to moisten the oats, the resulting thick suspension tended to glue the caryopses together, and this resulted in decreased reaction rates. The most effective method of adding oat lipase was simply to stir it into the reaction mixture after the oil had been added. Seemingly, the lipase must attach itself to the caryopsis surface, else there would not be water for the hydrolysis reaction to occur. There was little hydrolysis in reaction mixtures in which lipase was added without water. Commercial preparations of *C. rugosa* and hog pancreatic lipase also were effective in accelerating the initial rates of reaction when they were added to oat bioreactors.

Attempts were made to find oat varieties with high concentrations of lipase. Hammond and Frey (13) reported significant variation among varieties in lipase activity. The standard oat strain that we used, B605-1085, was related to a strain that they had found high in lipase activity. Using their screening method, we screened 1107 oat selections for lipase activity. Varieties identified as rich in lipase were tested in bioreactors. Significant differences were found, and the best strain that we identified(Y907-5-5) was about 45% better than the standard strain.

Rice bran also is known to be rich in lipases, but attempts to make a bioreactor from unpolished rice gave no hydrolysis. Lipases added to moistened rice, however, made effective bioreactors just as the oat caryopses did.

Effect of substrate on hydrolysis rate. The initial rate of hydrolysis of coconut oil was 1.3 times greater than that of soybean oil, that of castor oil and triolein was 0.6 times greater than that of soybean oil. These results il-

lustrate that the rate of hydrolysis can vary with the oil phase composition. The effects probably can be attributed to the specificity of the enzyme and the viscosity of the oil phase.

Product inhibition. We theorized that product inhibition caused the slowing of the rate of hydrolysis as hydrolysis increased. This was verified by adding moistened oats to partly hydrolyzed oil. The results are shown in Figure 3. Obviously the rates, as indicated by the slope of the plots, decreased as the free fatty acid concentration increased. When free oleic and linoleic acids were added to soybean oil, inhibitory effects similar to those obtained with partly hydrolyzed soybean oil were noted, and linoleic resulted in only 62% as much hydrolysis as oleic acid when they were added to the soybean oil at the same concentration. Figure 4 shows the inhibitory effect of glycerol dissolved in the water used to moisten the oats.

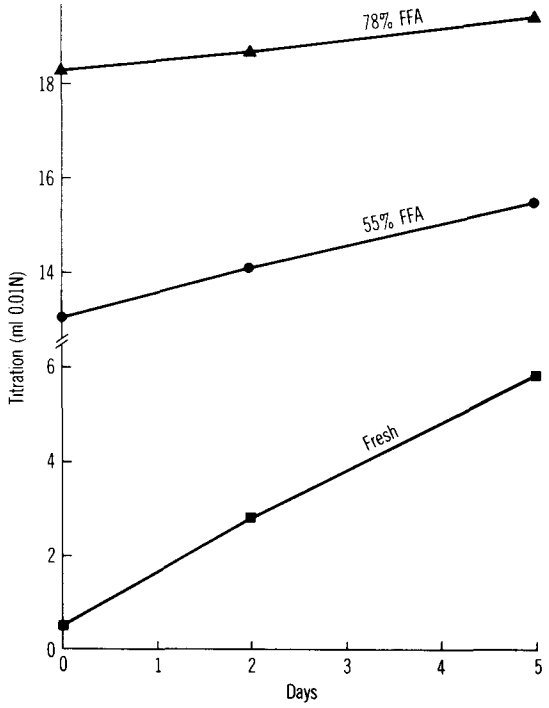

FIG. 3. The effect of free fatty acids on the hydrolysis of soybean oil by wet oat caryopses. Oil phases were fresh oil or oil previously hydrolyzed by oats to contain 55 or 78% free fatty acids. Conditions: 0.2 mL water/g caryopses, 36°C, no agitation.

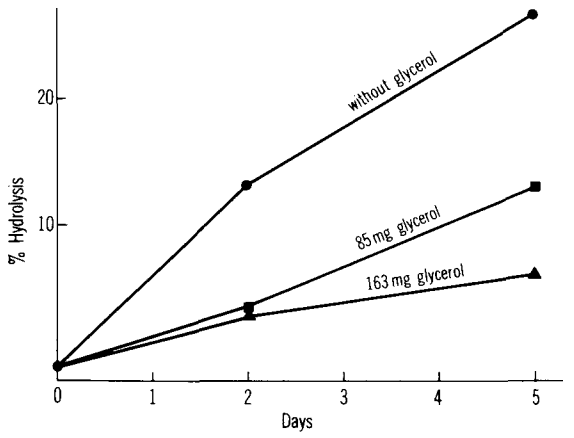

FIG. 4. The effect of glycerol on the hydrolysis of soybean oil by wet oat caryopses. Conditions: 0.2 mL water/g caryopses, 36°C, no agitation. The glycerol was added with the water, and the amounts indicated are per g caryopses. Complete hydrolysis of the soybean oil would yield 163 mg glycerol/g caryopses.

Attempts at high degree of hydrolysis. For oat bioreactors to be an attractive method of hydrolysis, the reaction must be almost complete. To get high conversions it is necessary to deal with the problem of product inhibition. One cannot decrease the oil/caryopses ratio more than about 1:1 by weight and keep the caryopses covered with oil, and lack of contact between oil and caryopses would reduce the reaction rate and allow mold growth. One solution is to replace oats that have become inhibited with product with fresh moistened caryopses. Although the system still is inhibited by the presence of free fatty acids, inhibition caused by the accumulation of glycerol is alleviated. Table 2 shows that, with three changes of oats, it was possilbe to obtain over 90% conversion in 58 days in unstirred conditions. These times can be diminished significantly if the substrate is diluted with hexane and agitated. Figure 2 shows that even without agitation, conversions of 70% could be obtained in 4 days when the oil phase contained 75% hexane. With 1,500 units of *C. rugosa* lipase/g caryopses and two changes of oats, 90% conversion was obtained in undiluted oil in 7 days in unstirred reactors. Thin-layer chromatograms showed that the triglyceride was completely exhausted at 90% conversion, and only free fatty acids and mono- and diglycerides were left.

Glycerol recovery. The glycerol released during fat hydrolysis is a valuable by-product. Over 90% of the released glycerol accumulated inside the oat caryopses. The rest was in the oil phase. The glycerol could be extracted from both the oats and the oil phase with water. Figure 5 shows the extraction of glycerol from whole oats with water. Considerable amounts of water were required to immerse the whole oats, and the glycerol was recovered as a very dilute solution.

Synthesis and transesterification with oat bioreactors. It was possible to demonstrate the incorporation of free fatty acids into oil by incubating moist oats with an equimolar mixture of the free fatty acids and soybean oil. For example, the amount of oleic acid in the triglyceride fraction was raised from 25.7 to 41.7% in this way. In

TABLE 2

The Extent of Hydrolysis of the Soybean Oil by Three Successive Batches of Fresh Oats[a]

	Reaction time (days)	% Hydrolysis
First batch	13	33.8
Second batch	26	60.3
Third batch	39	86.4
	58	91.3

[a]The reaction was carried out without agitation. For each reaction 15.5 g oil was used with 10 g oats. For the second and third batches, the hydrolyzed oils obtained by the previous batch were transferred to 10 g of fresh oats.

general, considerable amounts of water had to be added to make the oats effective catalysts, and this favored hydrolysis over synthesis.

Table 3 shows the synthesis of glycerol esters from oleic acid and glycerol by oat bioreactors. At 10 days, these reaction mixtures contained only mono- and diglycerides, but at 38 days, some triglyceride had been formed.

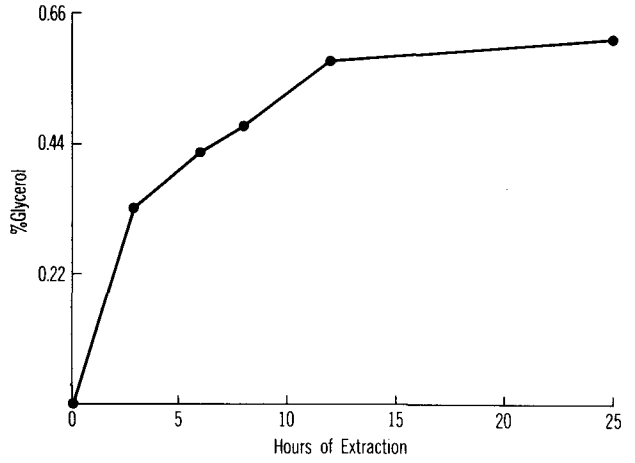

FIG. 5. The concentration of glycerol in water circulated at 60 mL/min through used oat caryopses after 26.5% hydrolysis of soybean oil.

TABLE 3

The Esterification of Oleic Acid with Glycerol in the Presence of Wet Oat Caryopses

	Sample 1	Sample 2	Sample 3
Oleic acid (nmoles/g oats)	6.0	6.0	6.0
Glycerol (nmol/g oats)	2.0	1.0	2.0
Water (nmole/g oats)	5.6	5.6	16.7
Meq. of oleic acid/g oil phase after 38 days	2.47	2.82	2.99
Percentage of fatty acid esterified[a]	28.5	19.3	14.4

[a]Determined by titration.

The ability of oat bioreactors to catalyze reactions with a number of alcohols was explored. Normal primary alcohols with a chain length greater than four were esterified, but no esters were formed when the alcohol was *iso*-pentyl, *tert*-pentyl, 4-octyl, phenol, phenylethanol, cyclohexanol, cholesterol, maltose, glucose, fructose, sucrose, or sorbitol.

Very good conversion of soybean oil to alkyl esters was observed when soybean oil-n-primary alcohol (1:3 by weight) was contacted with caryopses moistened with 0.2 mL of water/g. All triglyceride disappeared by 24 days, and only alkyl esters of the soybean oil fatty acids were observed. During the early stages of the reaction, free fatty acids were detected, but these eventually disappeared. The reaction mixture was not stopped by the accumulation of glycerol as it was with hydrolysis reactions. This may be because the excess alcohol binds the glycerol and keeps it from accumulating in the caryopses.

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