Lipase Immobilized on a Hydrophobic, Microporous Support for the Hydrolysis of Fats[1]

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Enzymatic hydrolysis of triglycerides has been studied as a low energy-consuming alternative to the present steam (Colgate-Emery) process. Cost analysis of the enzymatic process indicates that use of immobilized lipase compares favorably with the present steam process. This paper discusses the search for an adsorbent to use as the support material for the lipase. Hydrophobic microporous powders, membranes and fibers were found to give the best performance, as little of the lipase's activity was lost upon immobilization. Lipase immobilized on Accurel powder has been studied in various reactor configurations for the hydrolysis of triglycerides. Reactor designs studied include co-current and counter-current fixed beds, continuous stirred tank reactors, and the diaphragm reactor. Productivities of the latter two reactor types were 1100 and 1700 kg fatty acid per kg immobilized lipase.

The existing process used to split fats is the Colgate-Emery steam hydrolysis. Typical operating conditions are 250 C and 50 atm. Maintaining these reaction conditions makes the splitting operation very energy-intensive. Furthermore, the capital investment for steam splitting is high because a special splitter column that will withstand the high temperature and pressure as well as the corrosiveness of the fatty acid is needed. On the positive side, the steam process gives high conversions yielding a fatty acid stream that is 97+% split and a sweetwater stream containing 12% glycerol.

An alternative approach to steam hydrolysis is enzymatic splitting at milder conditions, 30-45 C and atmospheric pressure. The initial reason for studying enzymatic fat splitting was to lower energy costs by operating at ambient conditions. As the research has progressed, another advantage, improved product quality, has gained in importance. Not only is the color of the fatty acid lower, but the color of the sweetwater is improved dramatically. In some cases, the sweetwater is actually water-white.

A third advantage of enzymatic splitting is that the capital investment will be lower. For example, for a stirred tank reactor, the equipment costs would be less than for a steam splitter column. Finally, to be competitive enzymatic fat splitting must be able to produce yields comparable to or better than that of the steam process (viz. 97% split) or the engineers will not be interested in incorporating this new technology into the fatty acid manufacturing process.

The enzyme that catalyzes the hydrolysis of triglycerides to fatty acids is lipase (triacylglycerol acylhydrolase; EC 3.1.1.3). Because Akzo Chemicals Inc. is not an enzyme producer, part of the research effort on this process has been the evaluation of lipases available from enzyme suppliers. The ideal lipase for use in fat splitting would possess several characteristics. First, the lipase should be random. It should not demonstrate position or chain length selectivity. In other words, the lipase should hydrolyze a variety of fats and oils to completion.

Second, the lipase should be thermostable at 45-55 C. This condition is necessary for the use of tallow as a feedstock. Bleachable Fancy Tallow (BFT), one of our main feedstocks, has a titer of 41 C. A comfortable operating range above the melting point of BFT would be 45-55 C. This requirement has proven to be the most difficult one to meet. Most of the random lipases currently available are isolated from *Candida*. These enzymes are characterized by rapid thermal deactivation at temperatures above 42 C.

Third, to match the energy costs associated with steam splitting, the price of the lipase needs to be near $5/lb. While there are enzymes currently available in this price range, none of them is a lipase. In fact, lipase prices are an order of magnitude higher. Thus, at an early stage in the research, it was apparent that the lipase would have to be immobilized so that it could be reused several times. Each reuse of the catalyst would lower the lipase cost and simultaneously increase the feasibility of enzymatic splitting. To keep immobilization costs to a minimum, adsorption was selected as the immobilization technique.

TABLE 1

Lipase Supports

Support	$\frac{\%\text{ FA imm at 24 hr}}{\%\text{ FA sol at 24 hr}} \times 100\%$
Celite	16
Cellulose	24
Ethyl Cellulose	52
Silica Gel	23
Kieselguhr	30
Clay	13
Alumina	9
CPG-100	34
Carbon	12
Accurel, nylon	18
HDPE <150μ	98
HDPE 150-450μ	76
PP std grind	88
PP <250μ	107
Celgard 2500	85
Profax PP	14
Microthene HDPE	12

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EXPERIMENTAL PROCEDURES AND RESULTS

Many adsorbents were screened as immobilization supports for lipase. A standard procedure was developed and used for evaluating these materials. A lipase solution containing 6000 IUs of Enzeco lipase (Enzyme Development Corporation, New York, NY) in 100 ml of 0.1 M phosphate buffer at pH 7.0 was prepared. One gram of the adsorbent was added to the lipase solution and stirred for 60 min. Hydrophobic support material was prewet with 20-5 ml of ethanol before addition to the lipase solution. After immobilization, the support material was removed by vacuum filtration and rinsed with 300 ml of buffer to remove soluble lipase from the adsorbent. The activity of the immobilized lipase then was assayed in a batch hydrolysis of 50 ml of BFT and 100 ml of buffer at 42 C for 24 hr. Samples of the reaction mixture were taken during the assay and analyzed for fatty acid content by Gel Permeation Chromatography (GPC).

The Waters' GPC system used consisted of a U6K Injector, a Model 510 Pump, a Model 401 Refractive Index Detector, and three 100 Å μstyragel columns. The solvent used was tetrahydrofuran at a flowrate of 1.3 ml/min. In an analysis time of 20 min, the separation of triglyceride, diglyceride, monoglyceride and fatty acid peaks was obtained. The four peak heights then were multiplied by relative response factors and normalized to 100%. The resulting fatty acids levels (% FA) were used to monitor the progress of the BFT hydrolysis.

Table 1 lists the assay results for several support materials. The fatty acid acid levels at 24 hr are given as a ratio of the immobilized lipase result to that of a control assay done under the same conditions using 6000 IUs of soluble lipase. Thus, the ratio is an indication of the percent lipase activity retained upon immobilization. Most of the common enzyme supports were characterized by high losses of lipase activity upon immobilization. Surprisingly, Accurel powders made from high-density polyethylene (HDPE) and polypropylene (PP) were excellent materials for lipase. These powders, manufactured by Enka AG (Obernburg, West Germany), are microporous products that are available in a variety of forms besides powder, such as membranes and fibers (1,2). Celgard 2500, a microporous PP membrane manufactured by Celanese, also performed well. Hercules' Profax and USIs Microthene, which are non-porous, polymeric powders (PP and HDPE, respectively), did not perform well as lipase supports. Therefore, it was concluded that hydrophobic, microporous polymeric materials are superior adsorbents for lipase immobilization (3).

The behavior of lipase immobilized on Accurel has been characterized in several ways. Figure 1 shows the pH-activity curve for the immobilized lipase and the soluble lipase. The activity axis is actually the % FA at the one-hr mark in a batch BFT hydrolysis reaction similar to the one described above. The lipase's pH-activity curve did not shift upon immobilization. However, it was interesting that after one hr of reaction, the activity of the immobilized lipase actually was higher than that of the soluble control in the pH 4-7 range. Similar behavior was observed on the temperature-activity curve. The curve did not shift upon immobilization, but the immobilized lipase did perform better than the soluble at temperatures in the operable range.

The storage stability of lipase immobilized on Accurel powder also was investigated. Lipase was immo-

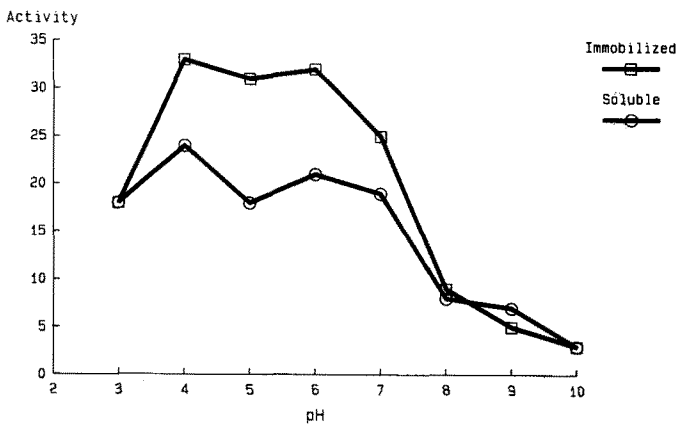

FIG. 1. PH-activity curve for *Candida* lipase immobilized on HDPE Accurel powder vs soluble lipase.

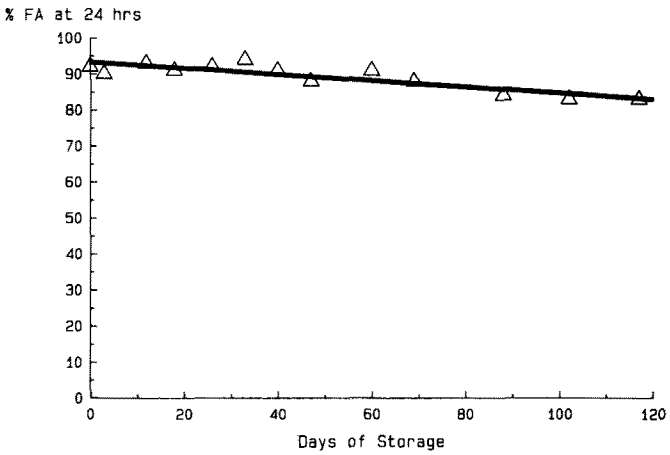

FIG. 2. Storage stability of *Candida* lipase immobilized on HDPE Accurel powder.

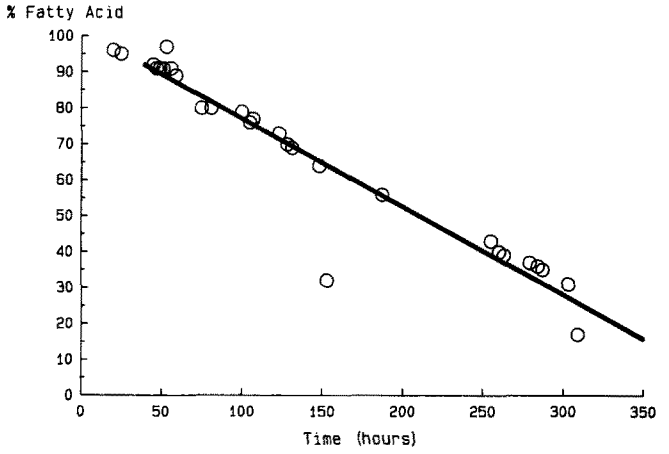

FIG. 3. Performance curve for continuous, co-current fixed bed column reactor. *Candida* lipase immobilized on HDPE Accurel powder; olive oil substrate.

bilized on 20 g of HDPE Accurel powder, air-dried at ambient conditions for three days, and stored on the shelf in a glass jar. Periodically, one gram of the immobilized lipase was assayed by the procedure given above on a BFT substrate. Figure 2 illustrates that after 120 days of storage, the immobilized lipase lost less than 10% of its original activity.

More important, the operational stability of the lipase-Accurel product has been evaluated in three continuous reactor designs: (a) the fixed bed column, (b) the diaphragm reactor and (c) the continuous stirred tank reactor (CSTR).

Fixed Bed Column. The first flow pattern studied in a fixed bed column was counter-current with olive oil fed to the bottom of the column and water fed to the top. This flow scheme is similar to that of the current steam splitter. The jacketed glass column was maintained at 40 C. The catalyst bed consisted of lipase immobilized on 10 g of HDPE Accurel granules, 2-3 mm in diameter. Dimensions of the bed were 2.5 cm diameter by 26 cm length. Flowrate of the oil ranged from 10-20 ml/hr. Flowrate of the buffer was 4-5 ml/hr. In fact, balancing the flowrates to maintain stable oil/water interfaces at the top and bottom of the fixed bed proved to be a major difficulty in the operation of the counter-current fixed bed reactor. Three reactors were run, each for a period of one day. The highest % FA obtained was only 34%, so co-current flow of the oil and water was investigated next.

Olive oil and buffer were fed to the bottom of a glass column using separate FMI Model RP-G6 Lab pumps. The flowrates were maintained at 5 and 15 ml/hr, respectively. The column jacket was maintained at 40 C by a Lauda Model C12 water bath. A glass frit at the bottom of the column supported the bed of immobilized lipase. Several forms of Accurel products were tried as lipase supports—PP melt-blown fibers, HDPE granules, and HDPE powder, 150-450 μ. The powder gave the best results: essentially complete conversion.

Figure 3 shows the conversion curve for such a co-current fixed bed reactor. The immobilized support for the reactor was prepared by dissolving 1.5 g of Enzeco lipase in 100 ml of 0.1 M phosphate buffer, pH 7.0, and adding 10 g of HDPE Accurel powder. Analysis using the Antek Model 707 Chemiluminescent Nitrogen Analyzer revealed that 60% of the lipase was adsorbed onto the powder. The immobilized enzyme then was placed into the jacketed column, forming a bed of 2.1 cm diameter by 18 cm length. Fatty acid and sweetwater exited together from the top of the column and were collected in a graduated cylinder. Samples of the effluent stream were analyzed daily by GPC to monitor the decay of the immobilized lipase. Exponential regression of the % FA vs time led to a half-life of 157 hr for the immobilized lipase.

A recurring problem with the fixed bed design for the two-phase substrate was that as the oil passed through the column, the oil droplets, initially dispersed by the glass frit at the entrance of the column, were coalescing. Large globules of the oil phase were visible in the catalyst bed. To improve catalyst performance, a way of redispersing these oil droplets was needed. One approach that was tried was to introduce additional frits at intermediate points in the bed. However, experiments with a column containing two additional frits spaced 4.5 cm demonstrated no improvement in performance over a control column containing only the entry frit. Perhaps the 4.5 cm spacing was too large. But additional frits increased the column backpressure beyond acceptable limits.

The effect of glycerol concentration on the immobilized lipase was studied in a series of three fixed bed reactors containing 0%, 20% and 40% glycerol in the buffer. These columns were run at room temperature on a 25/75 olive oil/buffer substrate that was mixed before entry to the column. The total flowrate was 17 ml/hr. The bed contained 10 g of the lipase-Accurel powder. The columns were in operation for 300-500 hr.

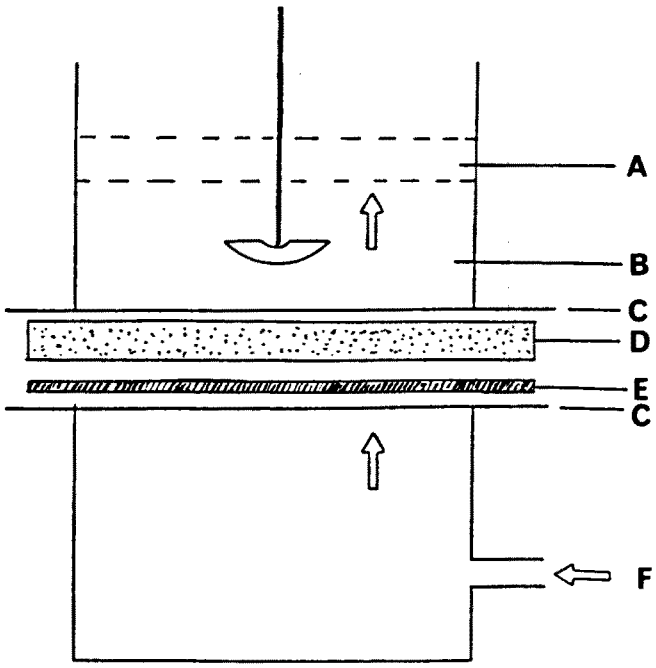

FIG. 4. Diaphragm reactor. A, fatty acid; B, buffer; C, support screen; D, lipase immobilized on PP Accurel fibers; E, filter cloth; F, olive oil.

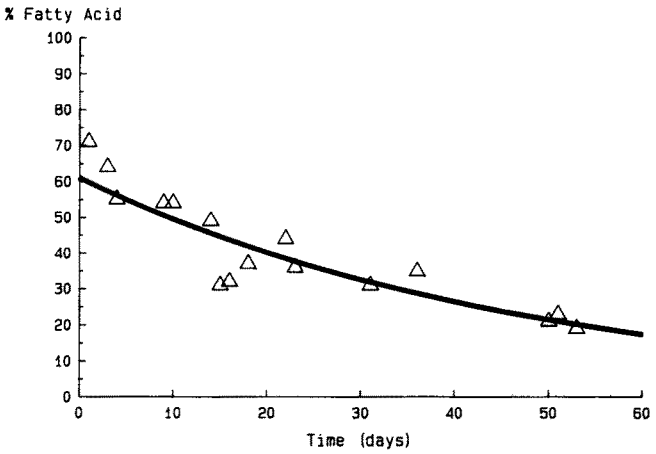

FIG. 5. Performance curve for semicontinuous diaphragm reactor. *Candida* lipase immobilized on PP Accurel fibers; olive oil substrate.

As expected, in the column containing 40% glycerol in the aqueous phase, the initial conversion dropped to only 45% FA. However, the half-life of the immobilized lipase increased dramatically with increasing glycerol concentration, as shown below:

% Glycerol	Half-life (hr)
0	110
20	220
40	1040

Stabilization of enzymes with glycerol is a well-known phenomenon. Furthermore, stabilization of lipase with glycerol has been reported (4).

Diaphragm Reactor. Use of membrane reactors for enzymatic fat splitting has been reported (4,5). The diaphragm reactor discussed in this section is a unique design, as depicted in Figure 4 (6). The reactor contains two reservoirs: a lower olive oil one and an upper water one. Oil is pumped into the lower reservoir. The oil is forced through the diaphragm comprised of two layers. The first layer is a polymeric filter cloth rated at 3–5 μ. The oil is dispersed into fine droplets as it passes through. The second layer of the diaphragm is a pad of PP Accurel fibers with lipase immobilized thereon. Thus, the finely dispersed oil droplets contact the lipase before having time to coalesce. The fatty acid then rises to the top of the buffer layer and can be withdrawn continuously from the top of the reactor.

To evaluate the operational stability of the immobilized lipase, the diaphragm reactor was run in a semi-continuous mode for approximately two months. The reactor was made from 15 cm plexiglass pipe. The diaphragm contained 5.0 g of melt-blown fibers that contained 0.3 g of Enzeco lipase. Each day, 450 ml of olive oil were fed into the reactor by gravity feed over a period of six hr. The upper reservoir initially contained 1800 ml of 0.05 M EDTA buffer at pH 7.0. At the end of six hr, the fatty acid layer on top of the upper reservoir was analyzed by GPC. Figure 5 shows the fatty acid conversions obtained over the course of the experiment. Exponential regression analysis of the data, with the time corrected to hours of continuous operation, led to a half-life estimate of 223 hr for the immobilized lipase.

Continuous Stirred Tank Reactor (CSTR). The schematic of the CSTR is in Figure 6. Oil and water were continuously pumped into the reactor from separate reservoirs. Agitation was supplied to the vessel by an overhead stirrer. The liquid level in the reactor was maintained by the use of a Therm-O-Watch controller, which activated a third pump whenever the reactor volume reached a preset level. The immobilized lipase powder mixed freely in the reactor but was retained in the reactor by a screen placed in the entry of the effluent take-off tube.

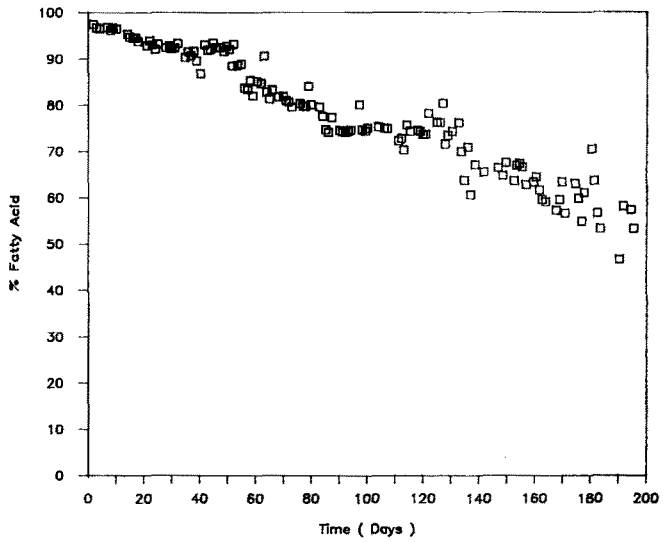

FIG. 7. Performance curve for CSTR. *Candida* lipase on HDPE Accurel powder; olive oil substrate.

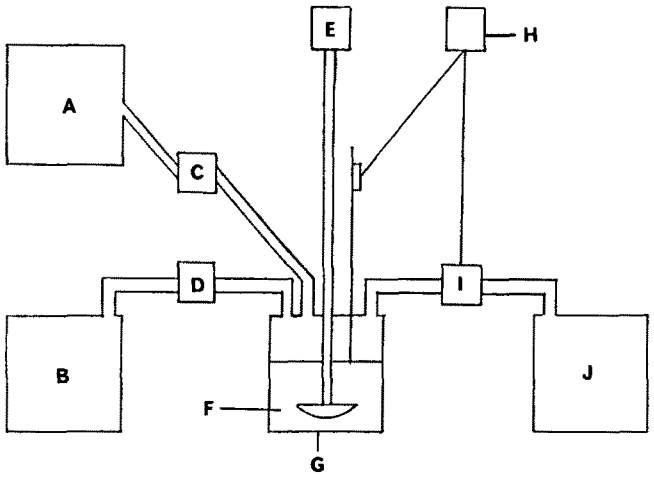

FIG. 6. CSTR. A, water reservoir; B, oil reservoir ; C, water pump; D, oil pump; E, overhead stirrer; F, lipase immobilized on HDPE Accurel powder; G, reactor; H, Therm-O-Watch controller; I, effluent pump; J, sweetwater and fatty acid collection reservoir.

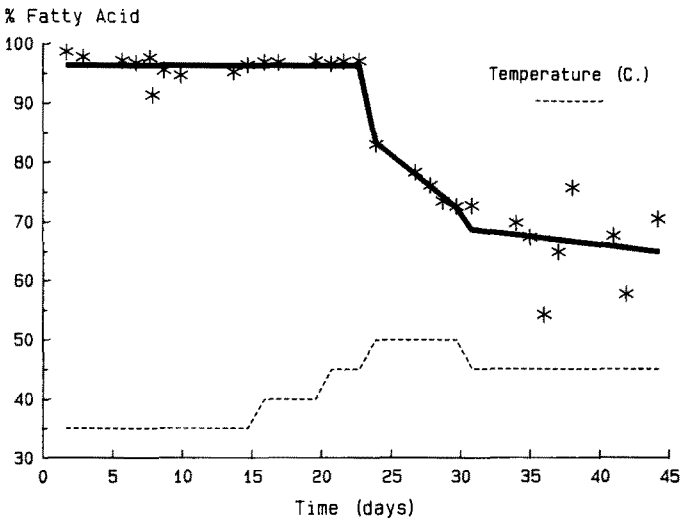

FIG. 8. Effect of temperature on performance of immobilized lipase in CSTR.

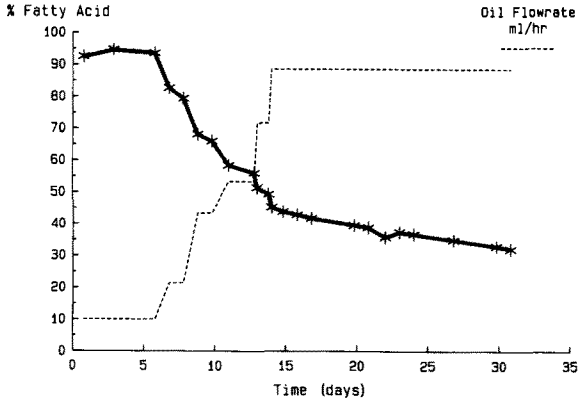

FIG. 9. Effect of olive oil flowrate on performance of immobilized lipase CSTR. Water flowrate = 12 ml/hr.

The best performance a CSTR on an olive oil substrate is shown in Figure 7. This CSTR contained 20 g of immobilized lipase in a reactor volume of 500 ml. Average flowrates were 4.7 ml/hr for olive oil and 6.3 ml/hr for water. The jacketed vessel was maintained at 35 C. The reactor was in continuous operation for more than 200 days. Exponential regression of the conversion data in Figure 7 gave a half-life of 237 days for the immobilized lipase.

The effects of several parameters have been studied in the CSTR. Figure 8 shows the effect of reactor temperature on the immobilized lipase. The substrate and setup of the CSTR were similar to that described above. However, the temperature was raised in 5 C increments, starting at 35 C. No effect on conversion was observed until 50 C was reached. At this temperature, deactivation of the immobilized lipase was rapid and irreversible. This behavior is expected, as *Candida* lipases are known to deactivate above 42 C.

Another variable studied was the effect of the olive oil flowrate on the fatty acid conversion level. The results of these experiments are shown in Figure 9. The water flowrate was held constant at 12 ml/hr. The oil flowrate was varied from 10–90 ml/hr. As expected, the conversion levels dropped with increasing oil flowrates.

DISCUSSION

In summary, to compare the three reactor designs—fixed bed, diaphragm and CSTR—productivities were calculated for each by integrating the conversion curve obtained on an olive oil substrate over a time period equal to two half-lives of the immobilized enzyme (IME). Summary data are:

Type	Half-life (hr)	Productivity (kg FA/kg IME)
Fixed bed	196	100
Diaphragm	223	1700
CSTR	5680	1100

The productivities of the CSTR and diaphragm reactors for enzymatic fat splitting lead to immobilized lipase costs that are competitive with present energy costs for steam splitting.

REFERENCES

1. Castro, A.J., U.S. Patent 4,247,498 (1981).
2. Castro, A.J., U.S. Patent 4,519,909 (1985).
3. Brady, C., L. Metcalfe, D. Slaboszewski, and D. Frank, U.S. Patent 4,629,742 (1986).
4. Hoq, M.,T. Yamane, S. Shimizu, T. Funada and S. Ishida, *J. Am. Oil Chem. Soc. 62*:1016 (1985).
5. Taylor, F., C. Panzer, J. Craig and D. O'Brien, *Biotechnol. Bioeng. 28*:1318 (1986).
6. Brady, C.,L. Metcalfe, D. Slaboszewski, and D. Frank, U.S. Patent 4,678,580 (1987).