REVIEW

Immobilized Lipase Reactors for Modification of Fats and Oils–A Review

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This review focuses on the use of immobilized lipase technology to effect hydrolysis, ester synthesis, and interesterification reactions. The various immobilization procedures, reactor configurations, and process considerations are all reviewed and discussed.

KEY WORDS: Hydrolysis, immobilized enzyme reactors, interesterification reactions.

Increased interest in the chemistry and biotechnology of fats and oils has emerged in recent years. This trend can be mainly attributed to the fact that oleochemicals are derived from renewable sources (e.g., vegetable oils and animal tallow). Hence they may be produced in virtually every country of the world (1). In addition, the increasing surplus of fats and oils in the more developed countries has bolstered both fundamental and applied research aimed at the manufacture of alternative lipid-derived products on an industrial scale (2–9). By virtue of their economic and ecological advantages, oleochemicals compete successfully with long chain acids, alcohols and their derivatives of petrochemical origin (10–17).

With recent advances in bioreactor technology and genetic engineering, many new and interesting ideas for employing biotechnology to produce oleochemicals from fats have been investigated. Among the most promising chemical routes of industrial interest are the hydrolysis, ester synthesis, and interesterification reactions of lipids brought about by lipases (10). Lipases selectively lower the activation energies of the chemical reactions they catalyze (18). With these enzymes, one can achieve much higher specificities and major enhancements in reaction rates relative to nonenzymatic reactions (19,20).

Total hydrolysis of ester bonds in triglycerides may be accomplished at high temperatures and pressures in the presence of steam, or alternatively at low temperatures by the action of a nonspecific lipase in the presence of excess water (2). The technical and economic feasibility of the enzymatic splitting of fats under mild conditions (21–31) relative to the uncatalyzed high-pressure countercurrent steam-splitting process employed in industrial practice is currently being investigated (32).

The high specificity of lipases towards triglyceride substrates with respect to the type and stereospecific position of the fatty acid residue has prompted a number of special applications within the food field. Flavors for use in foods for human and animal consumption have been changed and/or enhanced by the partial hydrolysis of triglycerides (33–38). The production of lipolyzed flavors deserves special mention (39). Commercial processes include the enzymatic modification of milkfat in milk (40,41), as well as the development of a number of enzyme preparations for use in the manufacture of Italian (42–43), American (44–48), and Cheddar cheeses (49,50). Examples of patented lipolyzed flavors and processes for their manufacture include lipolyzed milk compositions (51) and lipolyzed milkfat products, such as butter flavors (52–55), cultured cream flavors (56), Blue cheese flavors (57–59), and cheese-like flavors (60–62). The main features of lipolytic processes utilized commercially in the food industry have been discussed elsewhere (63–64).

Esterification reactions between polyhydric alcohols and free fatty acids are, in essence, the reverse of the hydrolysis reaction of the corresponding glyceride. The equilibrium between the forward and the reverse reactions is usually controlled by the water content of the reaction mixture. Examples of high-value chemicals obtained via use of lipases include the synthesis of oleic acid esters of primary and secondary aliphatic and terpenic alcohols (65,66), and the production of geranyl and menthyl esters from butyric acid and geraniol, or lauric acid and menthol, respectively (67).

The term interesterification refers to the exchange of acyl radicals between an ester and an acid (acidolysis), an ester and an alcohol (alcoholysis), or an ester and another ester (transesterification). Interesterification can be accomplished industrially by heating a mixture of the anhydrous ester and another potential reactant species at relatively high temperatures for a long time. As an alternative one may employ alkali metals or alkali metal alkylates at lower temperatures (2). The application of lipases for the modification of fats and oils by interesterification reactions is expected to become a parallel route to that involving the use of oil seed plants to produce oils and fats with desired characteristics (32,68–70). One example is the production of cocoa butter substitutes from cheaper feedstocks. This process can be accomplished via enzyme-catalyzed interesterification reactions involving: i) palm oil midfraction and stearic acid (71); ii) olive and safflower oils and stearic acid (72–74); and iii) palm oil midfraction and tristearoylglycerol (75–77).

IMMOBILIZATION OF LIPASES

Immobilization refers to the localization or confinement of a lipase. This process allows the lipase to be separated physically from the mixture of substrate and product for reuse (78).

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The choice of a carrier for the lipase is dependent upon several characteristics which are important in industrial processing applications. Among these factors are (79): mechanical strength; microbial resistance; thermal stability; chemical durability; chemical functionality; hydrophobic/hydrophilic character; ease of regeneration; loading capacity; and cost. However, no single substance simultaneously fulfills all these requirements in an optimal fashion (80). The selection of an immobilization strategy is based on process specifications for the catalyst, including such parameters as overall enzymatic activity, effectiveness of lipase utilization, deactivation and regeneration characteristics, cost of the immobilization procedure, toxicity of immobilization reagents, and the desired final properties of the immobilized lipase (81).

Numerous methods for achieving the immobilization of lipases are available, each involving a different degree of complexity and efficiency. The various methods used to date may be subdivided into two main categories (81): i) chemical methods, where covalent bonds are formed with the lipase; and ii) physical methods, where weaker interactions or mechanical containment of the lipase is utilized (81,82). Chemical methods include lipase attachment to a matrix by covalent bonds (83–96) and formation of a cross-linked lipase matrix (84,85). Physical methods include entrapment of the lipase within an insoluble gel matrix (67,79,89,90,93,97–101), containment of the lipase within porous hollow fibers (102,103) or microcapsules (104,105), adsorption of the enzyme on a carrier (67,71,75,85,86,90,100,102, 103,106–126), binding of the lipase to dried mycelia (127,128) or bacterial cell debris (129), and ion exchange between the enzyme and a support (95,96,130–136).

Support materials. The search for the most effective procedures for immobilizing lipases has led to the use of particular kinds of solid carriers. Some of the insoluble support materials commonly employed are porous glass and derivatives (67,85,86,89,90,100, 106,137); diatomaceous earth or kieselguhr (67,71,75,89,107,109,112,115,116,118,137–140); activated carbon (124); Duolite (89); cellulose and derivatives thereof (83,95,102,103,105,112,115,116, 121,137,141); silica and its derivatives (105,109,112,115,116,124,142,143); clay (112,115,116); kaolin (124); alumina (67,112,115,116,137,144); titania (67); stainless steel (84); nylon (112,115,116); polyethylene and derivatives (89,90,97,112,115,116); polypropylene and derivatives (89,90,97,100,115,116,119,145); polystyrene (96,105,115,116,122); poly-phenylparaphenylene oxide (115,116); acrylic copolymers (115,116); polyacrylamide (84,93,146); polyamide (94); enzacryl (137); polyurethane (67,89,90,100); Sepharose and derivatives thereof (83,85,93,123,126); Sephadex (83,95,113, 114,122,137); agarose and its derivatives (83,91,92,95,125,137); gelatin (147); alginate (100); Dowex (85,148); collagen (79,99,101); avicel (124); polyethylene glycol attached to magnetite (149); and fragments of fungal mycelia (127,128) or bacterial cell walls (129). Synthetic support materials have been manufactured or obtained in a number of forms and shapes, including membranes, fibers, granules, and powders.

Immobilization techniques. Physical adsorption is perhaps the most straightforward immobilization procedure. The preparation is easy, and the associated costs are small. The substrate specificity usually remains unchanged. Regeneration is often possible after treatment with detergents (126), strong denaturants (126), or wetting agents (115,116). Losses of enzyme in the process stream have been found to be significant when glycerol is present at high concentrations in the buffer phase (e.g., in ester synthesis reactions) (111,120). However, when a microporous hydrophobic support for lipase is employed, leakage of lipase may be made negligible, provided that the level of polyhydric alcohols is kept low (110,120,145,150) or even negligible (126). It is believed that this observation may be due to the quasi-irreversibility of the multisite protein adsorption process.

Immobilization by physical adsorption has been successfully achieved using flat membranes (99,101,110,111,119,120), a pad of fibers (112,115,116), and powders (67,85,89,90,100,106–109,112–118,121–125,138,140). Partial adsorption is believed to play an important role in addition to mechanical containment in the case of bundles of hollow fibers made of cellulose (102,103).

The adsorption of lipase onto membranes has been achieved by passing a buffered solution of the lipase through the membrane (102,103,119) or over the surface of the membrane (110,145) before starting the reaction, as well as by continuously contacting the membrane with lipase solution (111,120). The adsorption of lipase may be accomplished in an aqueous environment (103,119) or by soaking a hydrophobic membrane with fatty acids (102,111,120) or oil (111) prior to exposure to the solution of free lipase.

The standard procedure for immobilization of lipase on hydrophilic solid carriers in powder form consists of dissolving the lipase in a buffer solution, mixing this solution and the powder well (via stirring in a beaker or percolation through a packed column), removing the supernatant solution (by filtration or simple drainage), and possibly drying the support for storage. Among the supports that have been studied are Celite (67,71,89,107–109,112,115–118,138), Duolite (89), cellulose (102,103,112,115,116), ethyl cellulose (112,115,116), silica gel (112,115,116,124), kieselguhr (75,112,115,116,137), clay (112,115,116), kaolin (124), alumina (67,112,115,116,137), titania (67), nylon (112,115,116), Sepharose (85,123), Sephadex (113,114,122), activated carbon (124), avicel (124), and porous glass (67,86,90). Variations of the above procedure include immobilization by precipitating the lipase from a buffer solution with cold acetone, thus forcing it to adsorb on the surface of Celite particles, followed by vacuum drying (107,109,117,121,139,140). Lipases have also been immobilized by letting a buffer solution of lipase vaporize over Celite under reduced pressure (90,118).

Hydrophilic supports are generally characterized by high losses of lipase activity upon immobilization. These losses have been attributed to i) a change in the conformation of the lipase upon adsorption to a form which has reduced activity; ii) a situation in which only a small amount of lipase is immobilized, iii) a decrease in the ability of hydrophobic substrates to reach the active site of the lipase; or iv) the existence of steric

hindrance imposed by the carrier matrix which constrains flexibility of the lipase molecule.

In general, the experimental procedures for physical adsorption of lipases to hydrophobic supports are similar to those employed for hydrophilic supports. Variations include suspension of the support in powdered form in a water/ethanol mixture, degassing under vacuum, washing with water and buffer in a packed column, and percolation of the lipase solution through the column (86). Hydrophobic solid carriers, such as polypropylene powders, have also been prewet with polar solvents such as ethanol (112,115,116), isopropanol, methanol, acetone, and tetrahydrofuran (115,116) prior to the adsorption step. Use of polar solvents tends to decrease the time required for adsorption, although the absence of a prewetting step generally leads to higher activities for the immobilized lipase (115,116). Pretreatment with nonpolar solvents is disadvantageous (115,116). Among the hydrophobic supports studied are AE-cellulose derivatized with 2,4-toluene diisocyanate and bound to *n*-butanol, *n*-octanol, palmitic acid, and sebacic acid (141), palmitoyl cellulose (121), aliphaticamine (126), octyl (100), and phenyl derivatives of Sepharose (100,123), diaminododecane agarose (125), silicone-coated glass beads (85,106), an iodopropyl-derivative of porous glass (86), silicone-coated controlled-pore silica (109), high density polyethylene (112,115,116), polypropylene (112,115,116,145), styrene (115,116,122), polyphenylparaphenylene oxide (115,116), and acrylic copolymers (115,116).

The binding affinity of lipases to carriers possessing aliphatic chains is more specific towards even-numbered rather than odd-numbered carbon chain backbones, and almost negligible when the chain backbone has less than six consecutive carbon atoms (126). Use of microporous supports with a hydrophobic character (e.g., made from standard and high density polypropylene, or high density polyethylene) leads to very good performance for the immobilized lipase (112,115,116). The enhanced activity of lipase immobilized on such surfaces relative to that of the free enzyme has been attributed to a higher local concentration of substrate at the liquid/solid interface (106). In a detergent-free mixture of olive oil and buffer, the activity of lipase adsorbed on palmitoyl-cellulose was more than one order of magnitude higher than that of the free lipase. Binding of free fatty acids to the hydrophobic binding site seems to result in stimulation of lipase activity (121). Lipases immobilized by adsorption on supports that could make water available for hydrolysis performed satisfactorily in a reverse phase system (122). It has been shown that as the amount of lipase adsorbed per unit area (102,110,111,120) or unit weight (124,141) of support increases, the lipolytic activity tends to an asymptotic value. This fact suggests that diffusional limitations become important in lipase catalyzed reactions when high loadings of lipase are employed.

Adsorption of a lipase onto a support involves two basic steps—diffusion of the enzyme from the bulk of the solution to the surface of the support, followed by binding of the enzyme at available surface sites. The second order rate constant for the adsorption step has been reported to be 1.8×10^6 $M^{-1} \cdot s^{-1}$ (151). The binding step is often much faster than the diffusion step. Hence, the overall rate of adsorption tends to be diffusion controlled. Once equilibrium is reached, the amount of lipase adsorbed has traditionally been correlated in terms of a Langmuir isotherm (141,145,150–152). Experimental values of the binding constants and the surface area associated with each molecule are summarized in Table 1. The values for this surface area are an indication of the loading capacity of the support. Areas between 4,000 and 6,000 $Å^2$ are associated with each molecule for nonporous materials. The dissociation constants for the adsorption process vary between 10^{-8} and 10^{-6} M.

Entrapment of lipase entails capture of the enzyme within a matrix of a crosslinkable resin. For example, porcine pancreatic lipase has been immobilized by entrapment in polyacrylamide gel (146). When the number of coupling groups on the surface of the beads was increased, there was a concomitant increase in the specific activity of the bound enzyme. A lipase from *Humicola lanuginosa* was entrapped in a gel formed upon contact of sodium alginate with calcium chloride (100). A variety of lipases have been trapped using photo-crosslinkable resin prepolymers (89,90,97) and urethane prepolymers (67,89,90). The immobilization process consists of mixing the prepolymers with a photosensitizer (e.g., benzoin ethyl ether), melting, mixing with a lipase solution, and gelling by exposure to near ultraviolet radiation. An alternative method of entrapment consists of freezing a monomer solution containing the lipase in the form of small beads. Polymerization is then initiated using gamma radiation (98). Lipase can also be immobilized under nitrogen at room temperature by contacting the buffer solution of lipase with cyanogum and N,N,N′,N′-tetramethylethylenediamine, followed by the addition of ammonium persulphate (93). Lipases immobilized by entrapment display better activities and stabilities when the trapping resin is hydrophobic. The major disadvantage of this approach arises from mass transfer limitations imposed by the resin. Lipases have also been immobilized by entrapment within a collagen membrane containing liquid crystals (79). The preparatory suspension containing collagen fibrils and lipase powder was mixed with an ethanolic solution of 4-methoxybenzilidene-4′-n-butylaniline (79,99,101) or 4-cyano-4′-heptylbiphenyl (99,101). The suspension was cast and dried, and the membrane thus obtained was treated with glutaraldehyde (79).

Microencapsulation resembles entrapment, but in essence involves the formation of a dispersed solid phase consisting of very small beads or capsules. Microencapsulation of porcine pancreatic and microbial lipases has been claimed to produce a system which is stable when stored for up to three months (142,143). The process uses silicone dioxide or powdered dextran in a binder solution of ethylene maleic anhydride copolymer or dextrin. These compounds are dissolved together with the lipase in a mixture of ethanol and acetone. Following atomization in hot air, capsules with diameters of 10 to 20 μm were obtained. The effect of microencapsulation on the activity of lipase was also investigated (105). The lipase was enclosed in small, semipermeable capsules of polystyrene, silicone deriva-

TABLE 1

Thermodynamic Properties for Adsorption of Lipase

Dissociation constant	Area associated with each molecule	T [°C]	pH	Source of lipase	Support	Reference
n.a.[a]	5000[d]	25	8.6	Pancreas	Spherosil	86
1.2×10^{-6}[b]	n.a.	23	7.0	Hog pancreas	Sepharose	87
n.a.	9.17[f]	n.a.	6.0	*Aspergillus*	Celite	109
n.a.	1.11[e]	30	7.0	*Candida rugosa*	Sephadex	114
n.a.	5.18[f]	40	7.0	*Rhizopus arrhizus*	Celite	117
n.a.	0.000247[e]	25	7.5–8.5	*Thermomyces lanuginosus*	Acrylic	119
42.9[c]	0.153[e]	35	7.0	*Aspergillus niger*	Polypropylene	145
74.6–62.9[c]	0.233–0.315[e]	25–45	7.0	*Aspergillus niger*	Polypropylene	150
1.3×10^{-8}[b]	6000[d]	24	7.5	Hog pancreas	Siliconized glass	151
1.4×10^{-7}[b]	4500[d]	30	7.0	*Chromobacterium*	Siliconized glass	152
1.5×10^{-8}[b]	4375[d]	n.a.	n.a.	Dog pancreas	Siliconized glass	213

[a]Not available.
[b][M].
[c][$\mu g/cm^3$].
[d][$Å^2$/molecule].
[e][$cm^2_{support}/\mu g_{protein}$].
[f][$g_{support}/g_{protein}$].

tives, and ethyl cellulose. Microencapsulation of the enzyme was performed in an aqueous medium containing the lipase and a solution of the synthetic polymer in benzene. Capsules prepared under these conditions had diameters of 10^2–10^3 μm. The microencapsulated lipase was not able to attack large particulate substrates, presumably because of the limited permeability of the capsule wall to such substrates.

Microencapsulation of lipases has been studied for the accelerated ripening of cheese curds. This approach is particularly suitable for cheese processing because the immobilized enzymes can be kept separate from the cheese precursors until melting of the capsule occurs; capsules can be designed for maximum retention within the curd matrix by taking advantage of their charge, size, and pH sensitivity; and once entrapped, the encapsulated enzyme will be distributed within the raw material as bacterial cells would be (153,154). Controlled cheese-like flavor generation has been achieved using encapsulated lipases, cell-free extracts, spores, viable cells, or combinations thereof (155–165). Microcapsules have been prepared consisting of a coat of either unfractioned butterfat or a high melting fraction thereof; and a core, where the flavor producing materials are contained. This type of system provides a suitable microenvironment for the desired reactions within the capsule. The controlled hydrolytic action of lipases encapsulated in butterfat-coated microcapsules on the triglycerides of the coating has been successfully achieved (104). The free fatty acids released by this action were then oxidized (in the presence of molecular oxygen and co-encapsulated mold spores) to give methyl ketones that diffuse out of the capsules and enhance the flavor of blue cheese. Lipases have also been encapsulated in formaldehyde-treated gelatin, but difficulties were encountered in melting the capsule material to release the enzyme into mozzarella cheese at the desired temperature (147). It has been suggested that the use of higher melting fractions of milkfat may solve this problem (155).

Ion exchange resins consist of an insoluble material containing chemically bound charged groups and mobile counterions. These counterions can be reversibly exchanged with other ions with the same charge without any changes in the insoluble matrix. Since lipases are proteins, they may carry a charge depending on the pH of the solution and the types of amino acid groups constituting the protein. Thus lipases can be immobilized using ion exchange methods.

Lipases have been immobilized on diethylaminoethylcellulose (95) by percolating the buffer solution containing the lipase through a packed column at 5°C, and on anion exchange carriers, such as Duolite (131), at pH 6 using a long contact time. Carboxylic acid ion exchange resins performed successfully as supports for lipase (96). Macroporous ion exchange resins have also been used as efficient supports for lipases. These resins have the added advantage of giving reduced pressure drops for flow across the packing (130,132–136).

During cross-linking, the molecules of lipase are chemically linked with one another by the use of various bifunctional reagents. A common procedure consists of a preliminary step involving immobilization on an ion exchange resin (e.g., Dowex or Amberlite) in order to obtain a high loading of lipase, followed by one or more steps in which covalent bonds are formed. Examples of these steps include contacting the pretreated resin with glutaraldehyde dissolved in a suitable buffer in order to form a Shiff base as a product of reaction with amine residues (85,148), and treating with hydrogen sulfide solution to reduce the excess Shiff base and the excess of glutaraldehyde (148), or simply washing with phosphate buffer (85). Immobilization of lipase on Amberlite and Diaion was performed by cross-linking with hexamethylenediamine and glutaraldehyde the lipase adsorbed on the resins (100). Lipase has also been adsorbed on stainless steel beads using a process consisting of physical adsorption, followed by crosslinking with glutaraldehyde (84).

Covalent attachment involves derivatization of the support to provide a chemical group capable of reacting with available side groups of the proteinaceous backbone of the lipase. An example is the treatment of porous glass beads (85) or alumina (144) with γ-aminopropyltriethoxysilane in acetone, followed by reaction of the pretreated beads with glutaraldehyde solution, washing with buffer (to avoid crosslinking), and suspension in an aqueous solution containing the lipase. An alternative procedure consists of partially hydrolyzing polyamide with hydrochloric acid at high temperature, followed by treatment with glutaraldehyde, washing, and contacting with the lipase solution (94). Lipases have been immobilized to cyanogen bromide activated Sepharose using ethyl dimethylaminopropyl carbodiimide (85) and ethanolamine (93). The carbodiimide method has also been attempted with such supports as aminoalkyl derivatives of kieselguhr and glass, aminocaproin, wofatite, alanin derivatives of enzacryl and wofatite, carboxymethylcellulose, carboxylagarose, and CH- and AH-Sepharose (137). A similar procedure, including a colipase, has been used (87,88) with the derivatizing reagent dissolved in acetonitrile. Reports are available on the covalent immobilization of lipase on aminoethyl-cellulose and agarose (95) and on the binding of lipase to carboxylic acid ion exchange resins after conversion to the acid chloride form under slightly alkaline conditions (96).

Lipases have also been successfully immobilized to cyanogen bromide activated agarose beads (83,91,92,137), cellulose (83), and Sephadex (84). In order to avoid steric hindrance, the introduction of 4,4′-methylenedianiline as a spacer during attachment of lipase to Sepharose using the BrCN method has proved useful (83). Another procedure for attachment of lipases to agarose consists of first reacting the agarose beads with N,N′-ethylenediamine to obtain aminoethyl agarose. These transformed beads are allowed to react with succinic anhydride to yield succinylaminoethyl agarose, dehydrated with 1,4-dioxane, and reacted with N-hydroxy-succinimide and N,N′-dicyclohexylcarbodiimide before contacting with a buffered solution of lipase (91,92).

Immobilizations of lipase on polyacrylamide using a diazonium intermediate (84), on a polyaminopolystyrene resin by azo linkages (96), and to the alkylamine derivatives of controlled pore size glass and Spherosil via either glutaraldehyde or 1-cyclohexyl(2-morpholinoethyl) carbodiimide (89) have been reported. The diazonium intermediate has also been used to attach lipases to *p*-aminobenzylcellulose, enzacryl, poly-*p*-aminostyrol, and aminoacryl derivatives of kieselguhr (137). Similar procedures involving binding to the arylamine derivatives of glass and Spherosil by diazobinding have also been carried out (89,137). Covalent methods involving azide intermediates for supports such as carboxymethylcellulose, carboxymethyl-Sephadex, enzacryl, and wofatite have been reported (137).

A recent advance in covalent attachment of lipase during immobilization consists of providing the lipase with magnetic properties. This is done by coupling the lipase with 2,4-*bis*(O-methoxypolyethylene glycol)-6-chloro-s-triazine to obtain a polyethylene glycol-modified lipase, followed by addition of a solution of ferrous and ferric cations (149).

Binding of lipases to whole dried cells has been successfully employed for exogenous lipases produced by fungal species (127,128) or by bacteria (129). Fungal mycelia are used as a direct source of the enzyme, thereby eliminating the need for isolation and external immobilization procedures (127) in cases where the lipase is already bound to the cell wall. Batches of mycelia are grown in shake-flask culture, harvested, freeze-dried, defatted, ground in a knife mill, and stored *in vacuo* at room temperature until required for use (166). It has also been reported that an extracellular thermostable lipase produced by *Pseudomonas mephitica* var. *lipolytica* (a mesophilic bacterium) retains its activity in the supernatant fluid separated by centrifugation from a mixture obtained when fully grown cells are homogenized with small glass beads (under cooling provided by solid carbon dioxide) and disrupted with a sonic disintegrator (129).

One important factor that enables one to assess the effect of immobilization on the catalytic power of lipase is the fractional activity retained upon immobilization. Several values reported in the literature are compiled in Tables 2, 3, 4, 5, and 6. The range of these values is extremely wide (from much less than 1% to as much as 267%). Nonetheless, in most cases lipase loses a great deal of its intrinsic activity during immobilization. The activities of immobilized lipases range from a negligible value to 270 μmol of product per min per cm^2 of support when the latter is employed in membrane form. For cases where the support is employed in powdered form, activities may range from zero to 19,200 μmol of product per min per gram of support.

KINETICS OF THE ENZYMATIC REACTION

Several mechanisms have been proposed for lipase-catalyzed hydrolysis reactions (167). The vast majority of these mechanisms were developed for the case of soluble lipases acting on insoluble substrates (e.g., oil droplets dispersed in water). However, in the absence of diffusional limitations the validity of the aforementioned mechanisms may be easily extended to include the most complex case of having the lipase present in immobilized states.

The simplest kinetic model applied to describe lipase-catalyzed reactions is based on the classic Michaelis-Menten mechanism (168,169) as applied to emulsified oil/water systems. The kinetic steps can be represented schematically as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P + Q \qquad [1]$$

where E denotes the immobilized enzyme, S the substrate (glyceride), ES the enzyme-substrate complex, and P and Q the products (hydrolyzed glyceride and free fatty acid). The rate of formation of free fatty acids per unit volume of reacting fluid (r_v) can be represented in terms of this mechanism as:

TABLE 2

Hydrolysis Activity Retained after Immobilization of Lipases from *Candida* spp.

Activity after immobilization	Percent activity retained (%)	Source of lipase	Support	Method of binding	T (°C)	Reference
1.2[b]	n.a.	*C. cylindracea*	Sepharose	Covalent	37	85
0.07[b]	n.a.	*C. cylindracea*	Porous glass	Covalent	37	85
0.00[b]	n.a.	*C. cylindracea*	Dowex	Covalent	37	85
95[b]	107	*C. cylindracea*	Spherosil	Adsorption	n.a.	86
n.a.[a]	<1	*C. cylindracea*	Duolite	Adsorption	30	89
n.a.	21.7	*C. cylindracea*	Celite	Adsorption	30	89
n.a.	3.7	*C. cylindracea*	CPG-glutaraldehyde	Covalent	30	89
n.a.	5.6	*C. cylindracea*	CPG-carbodiimide	Covalent	30	89
n.a.	<1	*C. cylindracea*	CPG-periodic acid	Covalent	30	89
n.a.	1.1	*C. cylindracea*	Spherosyl-glutaraldehyde	Covalent	30	89
n.a.	12.5	*C. cylindracea*	Spherosyl-carbodiimide	Covalent	30	89
n.a.	1.5	*C. cylindracea*	Spherosyl-azo coupling	Covalent	30	89
n.a.	2.0	*C. cylindracea*	ENT	Covalent	30	89
n.a.	10.0	*C. cylindracea*	.7ENT + .3ENTP	Covalent	30	89
n.a.	14.7	*C. cylindracea*	ENTP	Covalent	30	89
n.a.	6.8–10.3	*C. cylindracea*	ENTP	Covalent	30	89
n.a.	<1	*C. cylindracea*	PU	Covalent	30	89
33–126[b]	29.0–30.8	*C. cylindracea*	Polyacrylamide	Entrapment	35	93
7.0–25.0[b]	24.1–29.2	*C. cylindracea*	Sepharose	Covalent	35	93
140.8–198.4[b]	n.a.	*C. rugosa*	ENT	Entrapment	35	97
252.8–302.4[b]	n.a.	*C. rugosa*	ENTP	Entrapment	35	97
19.1[c]	n.a.	*C. cylindracea*	Polypropylene	Adsorption	40	110
n.a.	16	*C. cylindracea*	Celite	Adsorption	42	112,115,116
n.a.	24	*C. cylindracea*	Cellulose	Adsorption	42	112,115,116
n.a.	52	*C. cylindracea*	Ethyl cellulose	Adsorption	42	112,115,116
n.a.	23	*C. cylindracea*	Silica gel	Adsorption	42	112,115,116
n.a.	30	*C. cylindracea*	Kieselguhr	Adsorption	42	112,115,116
n.a.	13	*C. cylindracea*	Clay	Adsorption	42	112,115,116
n.a.	9	*C. cylindracea*	Alumina	Adsorption	42	112,115,116
n.a.	34	*C. cylindracea*	CPG	Adsorption	42	112,115,116
n.a.	12	*C. cylindracea*	Carbon	Adsorption	42	112,115,116
n.a.	18	*C. cylindracea*	Nylon	Adsorption	42	112,115,116
n.a.	76–107	*C. cylindracea*	Porous HDPE	Adsorption	42	112,115,116
n.a.	12	*C. cylindracea*	Nonporous HPDE	Adsorption	42	112,115,116
n.a.	88	*C. cylindracea*	Porous PP std. grind	Adsorption	42	112,115,116
n.a.	14	*C. cylindracea*	Nonporous PP	Adsorption	42	112,115,116
n.a.	85	*C. cylindracea*	Porous PP membrane	Adsorption	42	112,115,116
n.a.	16–18	*C. rugosa*	Sephadex LH	Adsorption	n.a.	113,114
128[b]	n.a.	*C. rugosa*	Sephadex	Adsorption	30	114
n.a.	49.4	*C. cylindracea*	Sepharose	Adsorption	35	123
n.a.	99.6	*C. cylindracea*	Octyl-Sepharose	Adsorption	35	123
n.a.	96.8	*C. cylindracea*	Phenyl-Sepharose	Adsorption	35	123
21.9[b]	97.4	*C. cylindracea*	Kaolin	Adsorption	5	124
20.0–25.0[b]	84.2–97.3	*C. cylindracea*	Silica gel	Adsorption	5	124
22.5[b]	92.1	*C. cylindracea*	Activated carbon	Adsorption	5	124
20.6[b]	92.1	*C. cylindracea*	Avicel	Adsorption	5	124

Abbreviations: CPG: controlled pore glass, ENTP: polypropylene glycol, ENT: polyethylene glycol, HDPE: high density polyethylene, PP: polypropylene, PU: polyurethane.

[a]Not available.

[b]$\mu mol_{product}/(min \bullet g_{support})$.

[c]$\mu mol_{product}/(min \bullet cm^2_{support})$.

$$r_v = \frac{v_{max}[S]}{K_m + [S]}$$

$$v_{max} = k_{cat}[E]_{tot} \qquad [2]$$

$$K_m = \frac{k_{cat} + k_{-1}}{k_1}$$

where v_{max} is the rate observed when the lipase is saturated with substrate, K_m is the Michaelis-Menten constant, the brackets denote molar concentrations of the various species, and the subscript *tot* denotes the overall amount. Several values for v_{max} and K_m reported in the literature are tabulated in Table 7.

According to the above model, the apparent value of the parameter K_m should decrease as the physical dimensions of the emulsion droplets in suspension decrease. Similarly, the value of the parameter v_{max} should

TABLE 3

Hydrolysis Activity Retained After Immobilization of the Lipase from *Geotrichum* spp.

Activity after immobilization [$\mu mol_{prod}/(min \cdot g_{prot})$]	Percent activity retained (%)	Source of lipase	Support	Method of binding	T (°C)	Reference
18.5	66	*G. candidum*	ACMC	Covalent	35	137
6.84	25	*G. candidum*	CDICMC	Covalent	35	137
16.4	58	*G. candidum*	APABC	Covalent	35	137
5.04	18	*G. candidum*	ACMS	Covalent	35	137
7.02	25	*G. candidum*	ACA	Covalent	35	137
6.06	21	*G. candidum*	BS	Covalent	35	137
7.74	28	*G. candidum*	CCHS	Covalent	35	137
3.42	13	*G. candidum*	CAHS	Covalent	35	137
5.64	20	*G. candidum*	AAHS	Covalent	35	137
4.20	15	*G. candidum*	AEAA	Covalent	35	137
0	0	*G. candidum*	AEAH	Covalent	35	137
2.64	10	*G. candidum*	CEAC	Covalent	35	137
4.74	17	*G. candidum*	CEA	Covalent	35	137
0	0	*G. candidum*	EPTL	Covalent	35	137
6.24	22	*G. candidum*	AEPA	Covalent	35	137
3.42	12	*G. candidum*	APPAS	Covalent	35	137
8.04	28	*G. candidum*	AWCA	Covalent	35	137
1.62	6	*G. candidum*	AWAA	Covalent	35	137
19.0	67	*G. candidum*	CWMC	Covalent	35	137
0	0	*G. candidum*	AWMC	Covalent	35	137
7.62	27	*G. candidum*	AWAAMC	Covalent	35	137
2.76	10	*G. candidum*	CWMCA	Covalent	35	137
2.04	7	*G. candidum*	CWAC	Covalent	35	137
4.26	15	*G. candidum*	ASS	Covalent	35	137
0	0	*G. candidum*	Kieselguhr	Adsorption	35	137
2.52	9	*G. candidum*	Alumina	Adsorption	35	137
4.02	14	*G. candidum*	CKAD	Covalent	35	137
5.64	20	*G. candidum*	ALKAD	Covalent	35	137
6.24	22	*G. candidum*	AZKAD	Covalent	35	137
4.5	16	*G. candidum*	CAAD	Covalent	35	137
5.22	18	*G. candidum*	ALAAD	Covalent	35	137
5.22	18	*G. candidum*	AZAAD	Covalent	35	137
1.68–2.88	6–10	*G. candidum*	CAAG	Covalent	35	137
3.90–4.50	14–16	*G. candidum*	AAAG	Covalent	35	137

Abbreviations: AAAG: azo-aminoaryl glass, AAHS: aldehyde-AH-Sepharose, ACA: carbodiimide-carboxyl-agarose, ACMC: azide-carboxymethylcellulose, ACMS: azide-carboxymethyl-Sephadex, AEAA: azo-enzacryl AA, AEAH: azid-enzacryl AH, AEPA: aldehyde-enzacrylpolyacetal, ALAAD: aldehyde-alumina-aminoalkyl derivative, ALKAD: aldehyde-kieselguhr-aminoalkyl derivative, APABC: azo-carboxymethylcellulose, APPAS: azopoly-*p*-aminostyrol, ASS: aldehyde-servachrom-S-EMA 61, AWAA: azo-wofatit CA-aminoacryl derivative, AWAAMC: azo-wofatitMC-aminoacryl derivative, AWCA: azide-wofatit CA, AWMC: aldehyde-wofatit MC, AZAAD: azoaluminaaminoaryl derivative, AZKAD: azo-kieselguhr-aminoacryl derivative, BS: bromocyanide-Sepharose, CAAD: carbodiimide-alumina-aminoalkyl derivative, CAAG: carbodiimide-aminoalkyl glass, CAHS: carbodiimide-AH-Sepharose, CCHS: carbodiimide-CH-Sepharose, CDICMC: Carbodiimide-carboxymethylcellulose, CEA: carbodiimide-enzacrylalanine, CEAC: carbodiimide-enzacrylaminocaproine, CKAD: carbodiimide-kieselguhr-aminoalkyl derivative, CWAC: carbodiimide-wofatit MC-aminocaproin, CWMC: carbodiimide-wofatit MC, CWMCA: carbodiimide-wofatit MC-alanin.

increase as the bulk lipase concentration increases (170). However, if the substrate concentration is expressed as area/volume rather than as weight/volume, the plots of the reaction rate vs. substrate "concentration" for different emulsions of the same substrate coincide, and one obtains a single value for K_m (hereafter denoted by K_m') which is independent of the degree of dispersion of the substrate (170). For three types of supports, i.e., glass coated with olive oil, glass coated with paraffin, and glass coated with silicone, double reciprocal plots lead one to find values of 50.0, 67.8, and 58.0 cm for K_m' for assays of hydrolysis of olive oil emulsions catalyzed by a lipase from *Chromobacterium* at 30°C and pH 7 (152).

In the case of feedstocks from natural origin which contain more than one chemical species susceptible to lipase action (e.g., butterfat), the Michaelis-Menten mechanism denoted as Equation [1] may be extended in order to include competitive inhibition by every substrate, S_i, with respect to each other. For extents of hydrolysis below 70%, a pseudo-zero order rate expression arises which takes the form

$$r_{v,i} = \frac{v_{max,i}\,[S_i]}{\sum_{j=1}^{N} [S_i]} \qquad [3]$$

$$v_{max,i} = k_{cat,i}\,[E]_{tot}$$

The above equation is based on the following assumptions: i) the Michaelis-Menten constants, $K_{m,i}$, associ-

TABLE 4

Hydrolysis Activity Retained After Immobilization of Lipases from Various Sources

Activity after immobilization	Percent activity retained (%)	Source of lipase	Support	Method of binding	T (°C)	Reference
290[b]	n.a.[a]	*Pseudomonas*	MBBA	Entrapment	37	79
150[b]	n.a.	*P.*	Collagen	Entrapment	37	79
0.78[b]	n.a.	Hog pancreas	Sepharose	Covalent	37	85
0.05[b]	n.a.	Hog pancreas	Porous glass	Covalent	37	85
0.00[b]	n.a.	Hog pancreas	Dowex	Covalent	37	85
0.86[b]	n.a.	*Aspergillus niger*	Sepharose	Covalent	37	85
0.09[b]	n.a.	*A. niger*	Porous glass	Covalent	37	85
0.04[b]	n.a.	*A. niger*	Dowex	Covalent	37	85
2.1[b]	n.a.	*Rhizopus japonica*	Sepharose	Covalent	37	85
1.0[b]	n.a.	*R. japonica*	Porous glass	Covalent	37	85
0.1[b]	n.a.	*R. japonica*	Dowex	Covalent	37	85
4.0[b]	n.a.	*R. delemar*	Sepharose	Covalent	37	85
1.1[b]	n.a.	*R. delemar*	Porous glass	Covalent	37	85
0.07[b]	n.a.	*R. delemar*	Dowex	Covalent	37	85
16[b]	17	Pancreas	Spherosil	Adsorption	n.a.	86
3-1030[b]	9-150	Pancreas	Polyamide	Covalent	35	94
0.03-680[b]	1-117	Cotton seed	Polyamide	Covalent	35	94
1.2[b]	3.62×10^{-4}	*Mucor javanicus*	AEC	Covalent	37	95
24.1[b]	7.26×10^{-3}	*M. javanicus*	Agarose	Covalent	37	95
250-285[b]	8.1×10^{-2}	*M. javanicus*	DEAEC	Ion exchange	37	95
5.9[b]	1.8×10^{-3}	*M. javanicus*	QAES	Ion exchange	37	95
n.a.	36.6	*Rhizopus*	.2L+.4AA+.4ACa	Entrapment	30	98
n.a.	39.8	*R.*	.2L+.23AA+.2ANa+.4ACa	Entrapment	30	98
n.a.	14.5	*R.*	.2L+.39ANa+.39ACa+.02DS	Entrapment	30	98
n.a.	42.5	*R.*	.17L+.17%AA+.17%ANa +.17%ACa+.33%P	Entrapment	30	98
n.a.	4.8	*R.*	.2L+.58AA+.2P+.02DS	Entrapment	30	98
n.a.	13.5	*R.*	.2L+.2ANa+.2ACa +.38HEMA+.02DS	Entrapment	30	98
n.a.	267	*Mucor*	MBBA, CHBP	Entrapment	37	99
n.a.	63.7-84.4	*Hamicola lanuginosa*	ENTP	Entrapment	45	100
n.a.	0.9-13.8	*H. lanuginosa*	PU	Entrapment	45	100
n.a.	90.7	*H. lanuginosa*	Ca-alginate	Entrapment	45	100
n.a.	35.4-66.5	*H. lanuginosa*	Amberlite	Cross-linking	45	100
n.a.	33.7-53.4	*H. lanuginosa*	AACPG	Covalent	45	100
n.a.	10.7	*H. lanuginosa*	Octlyl-Sepharose	Adsorption	45	100
n.a.	54.4	*H. lanuginosa*	Diaion	Cross-linking	45	100
120-270[c]	n.a.	*Mucor*	MBBA, CHBP	Entrapment	37	101
n.a.	14	*Candida rugosa*	Cellulose	Adsorption	30	103
12000[b]	n.a.	*Aspergillus*	Celite	Adsorption	n.a.	109
19200[b]	n.a.	*Rhizopus arrhizus*	Celite	Adsorption	40	117
0.15[c]	0.4-0.9	*Thermomyces lanuginosus*	Acrylic	Adsorption	50	119
15[b]	200	*Chromobacterium viscosum*	Palmitoyl cellulose	Adsorption	n.a.	121
n.a.	35	*Pseudomonas mephitica*	DADA	Adsorption	n.a.	125
0.0[d]	n.a.	*Pseudomonas mephitica*	Butylamine-Sepharose	Adsorption	60	126
0.0[d]	n.a.	*P. mephitica*	Hexylamine-Sepharose	Adsorption	60	126
0.0[d]	n.a.	*P. mephitica*	Heptylamine-Sepharose	Adsorption	60	126
52.3[d]	n.a.	*P. mephitica*	Octylamine-Sepharose	Adsorption	60	126
16.2[d]	n.a.	*P. mephitica*	Nonylamine-Sepharose	Adsorption	60	126
106.5[d]	n.a.	*P. mephitica*	Decylamine-Sepharose	Adsorption	60	126
80.5[d]	n.a.	*P. mephitica*	Undecylamine-Sepharose	Adsorption	60	126
112.0[d]	n.a.	*P. mephitica*	Dodecylamine-Sepharose	Adsorption	60	126
79.4[d]	n.a.	*P. mephitica*	Tridecylamine-Sepharose	Adsorption	60	126
1005[b]	83	*P. mephitica*	Bacterial cell debris	Cell binding	60	129
720[b]	n.a.	*Aspergillus*	Filtercel	Adsorption	n.a.	138
990[b]	n.a.	*A.*	Hyflo Supercel	Adsorption	n.a.	138
n.a.	32	*Rhizopus microsporus-IC*	AE-cellulose	Adsorption	35	141
n.a.	13	*R. microsporus-EC*	AE-cellulose	Adsorption	35	141
n.a.	23	*R. oryzae*	Alumina	Covalent	50	144
0.101[c]	14.5	*Aspergillus niger*	Polypropylene	Adsorption	35	145
3.52[b]	n.a.	Hog pancreas	Polyacrylamide	Covalent	n.a.	146
n.a.	176	*Chromobacterium*	Glass + liquid paraffin	Adsorption	n.a.	152

TABLE 4 (continued)

Abbreviations: AA: 23% acrylamide + 1.3% NN′ methylene *bis* acrylamide (aq); AACPG: alkylamine derivative of controlled pore glass, ACa: 30% calcium acrylate (aq), AEC: aminoethylcellulose, ANa: 30% sodium acrylate (aq), CHBP: 4-cyano-4′-heptylbiphenyl, DADA: 1,12-diamino-dodecane agarose, DEAEC: diethylaminoethyl-cellulose, DS: divinyl sulfone, ENTP: polypropylene glycol, HEMA: 30% hydroxyethylmethacrylate (aq), L: lipase absorbed in Sephadex and dispersed in water, MBBA: 4-methyoxybenzilidene-4′-n-butylaniline, P: 20% 1-vinyl-2-pyrrolidone (aq), PU: polyurethane, QAES: diethyl-2-hydroxypropyl-aminoethyl-Sephadex.
[a]Not available.
[b]$\mu mol_{product}/(min \cdot g_{support})$.
[c]$\mu mol_{product}/(min \cdot cm^2_{support})$.
[d]$\mu mol_{product}/(min \cdot cm^3_{support})$.

TABLE 5

Ester Synthesis Activity Retained After Immobilization of the Lipase

Activity after immobilization	Percent activity retained (%)	Source of lipase	Support	Method of binding	T (°C)	Reference
0.9–1.5[b]	41–70	*Candida cylindracea*	Spherosil XOB	Adsorption	n.a.	67
1.3[b]	59	*C. cylindracea*	Porous glass	Adsorption	n.a.	67
1.3[b]	60	*C. cylindracea*	Alumina	Adsorption	n.a.	67
0.5[b]	22	*C. cylindracea*	Titania	Adsorption	n.a.	67
1.8[b]	82	*C. cylindracea*	Celite	Adsorption	n.a.	67
1.1[b]	52	*C. cylindracea*	Polyurethane	Entrapment	n.a.	67
0.021[c]	n.a.[a]	*C. rugosa*	Cellulose	Adsorption	25	102
5.5[c]	n.a.	*Rhizopus delemar*	Polypropylene	Adsorption	40	111
15.1[c]	n.a.	*Pseudomonas fluorescens*	Polypropylene	Adsorption	40	111
22.0[c]	n.a.	*Mucor miehei*	Polypropylene	Adsorption	40	111
44.1[c]	n.a.	*Chromobacterium viscosum*	Polypropylene	Adsorption	40	111
34.2[b]	n.a.	*Mucor miehei*	Resin	Ion exchange	60	133

[a]Not available.
[b]$\mu mol_{product}/(min \cdot g_{support})$.
[c]$\mu mol_{product}/(min \cdot cm^2_{support})$.

TABLE 6

Interesterification Activity Retained After Immobilization of the Lipase

Activity after immobilization [$\mu mol_{product}/(min \cdot g_{support})$]	Percent activity retained (%)	Source of lipase	Support	Method of binding	T (°C)	Reference
n.a.[a]	16	*R. delemar*	Spherosil XOB 15	Adsorption	40	90
n.a.	28	*R. delemar*	Spherosil QMA	Ion exchange	40	90
n.a.	9	*R. delemar*	Spherosil XOB 15	Covalent	40	90
n.a.	13–20	*R. delemar*	ENT	Entrapment	40	90
n.a.	29–82	*R. delemar*	ENTP	Entrapment	40	90
n.a.	3–19	*R. delemar*	PU	Entrapment	40	90
106	n.a.	*Aspergillus*	Celite	Adsorption	n.a.	109
320	n.a.	*Rhizopus arrhizus*	Celite	Adsorption	40	117
n.a.	85–90	*R. arrhizus*	Celite	Adsorption	n.a.	118
20	n.a.	*Mucor miehei*	Resin	Ion exchange	60	132
120	13	*Aspergillus*	Controlled pore silica	Adsorption	n.a.	138
63	n.a.	*A.*	Filtercel	Adsorption	n.a.	138
68	n.a.	*A.*	Hyflo Supercel	Adsorption	n.a.	138
32–860	n.a.	*A.*	Celite 560	Adsorption	n.a.	138

Abbreviations: ENT: polyethylene glycol, ENTP: polypropylene glycol, PU: polyurethane.
[a]Not available.

ated with every substate S_i (out of N possible substrates) are approximately equal; and ii) all $[S_i]/K_{mi}$ are very large compared to unity (145).

Numerous modifications of the aforementioned Michaelis-Menten mechanism have been proposed. For example, adsorbed lipolytic enzymes may irreversibly denature quite readily at the interface. This denaturation will then introduce an irreversible, first order step which converts the adsorbed enzyme to an inactive form, a process which competes with formation of the

TABLE 7

Apparent v_{max} and K_m Values for Lipolytic Reactions Catalyzed by Immobilized Lipases

v_{max}	K_m	T [°C]	pH	Source of lipase	Support	Method of immobilization	Solvent or continuous phase	Substrate	Reference
430–1470[b]	2.6[c]	37	8.5	*Pseudomonas*	MBBA	Entrapment	TMAC	SMPOE	79
0.80–2.52[b]	0.063[c]	25	9.0	Pancreatic	Steel	Crosslinking	W	TB	84
0.564[b]	0.896[c]	25	9.0	Pancreatic	PA	Covalent	W	TB	84
n.a.[a]	2.5[c]	45	8.5	Microbial	Agarose	Covalent	W	Olive oil	91
n.a.	2.5[c]	45	8.0	Microbial	Agarose	Covalent	W	Butter oil	91
n.a.	2.5[c]	37	8.2	Microbial	Agarose	Covalent	W	Olive oil	92
n.a.	6.0[c]	37	7.5	Microbial	Agarose	Covalent	W	Butter oil	92
n.a.	50[d]	35	7.5	*Candida cylindracea*	Polyacrylamide	Entrapment	W	Olive oil	93
n.a.	20[d]	35	7.5	*C. cylindracea*	Agarose	Covalent	W	Olive oil	93
1030[b]	n.a.	35	9.5	Pancreatic	Polyamide	Covalent	W	Triacetin	94
680[b]	n.a.	35	9.5	Cotton seed	Polyamide	Covalent	W	Triacetin	94
38[b]	n.a.	35	8.8	Pancreatic	Polyamide	Covalent	W	Tributyrin	94
8[b]	n.a.	35	8.8	Cotton seed	Polyamide	Covalent	W	Tributyrin	94
3[b]	n.a.	35	8.8	Pancreatic	Polyamide	Covalent	W	CSO	94
0.03[b]	n.a.	35	8.8	Cotton seed	Polyamide	Covalent	W	CSO	94
n.a.	26.6[c]	37	7.0	*Mucor javanicus*	DEAE-cellulose	Ion exchange	W	Olive oil	95
41.5[b]	0.043[c]	35	6.0	*Candida rugosa*	PEG	Entrapment	IO+20%W	Olive oil	97
62.5[b]	0.043[c]	35	6.0	*C. rugosa*	PPG	Entrapment	IO+20%W	Olive oil	97
97.5[b]	1.6[c]	20	n.a.	*Rhizopus arrhizus*	Mycelia	Cell binding	DIPE+0.7%W	Olive oil	127
87.0[b]	3.9[c]	35	n.a.	*R. arrhizus*	Mycelia	Cell binding	DIPE+0.7%W	Olive oil	127
82.2[b]	2.7[c]	50	n.a.	*R. arrhizus*	Mycelia	Cell binding	DIPE+0.7%W	Olive oil	127
268.4[b]	13.2[c]	20	n.a.	*R. arrhizus*	Mycelia	Cell binding	DIPE+0.17%W	Olive oil	127
341.8[b]	15.5[c]	35	n.a.	*R. arrhizus*	Mycelia	Cell binding	DIPE+0.17%W	Olive oil	127
269.9[b]	9.2[c]	50	n.a.	*R. arrhizus*	Mycelia	Cell binding	DIPE+0.17%W	Olive oil	127
97.0[b]	6.9[c]	20	n.a.	*R. arrhizus*	Mycelia	Cell binding	DIPE+0.37%W	Olive oil	127
136.6[b]	5.5[c]	35	n.a.	*R. arrhizus*	Mycelia	Cell binding	DIPE+0.37%W	Olive oil	127
116.6[b]	4.4[c]	50	n.a.	*R. arrhizus*	Mycelia	Cell binding	DIPE+0.37%W	Olive oil	127
n.a.	0.50[d]	35	8.5	*Geotrichum candidum*	CMC	Covalent	W	Olive oil	137
n.a.	0.48[d]	35	8.5	*G. candidum*	PABC	Covalent	W	Olive oil	137
0.00454[e]	63.6[d]	50	8.7	*Rhizopus oryzae*	Alumina	Covalent	W	TB	144
0.0625[e]	2.62[d]	50	8.7	*R. oryzae*	Alumina	Covalent	W	TB+PVA	144
0.101[f]	n.a.	35	7.0	*Aspergillus niger*	Polypropylene	Adsorption	W	Butter oil	145

Abbreviations: CMC: carboxymethylcellulose, CSO: cotton seed oil, DIPE: di-isopropyl ether, IO: isooctane, MBBA: 4-methoxybenzilidene-4′-n-butylaniline, PA: polyacrylamide, PABC: *p*-aminobenzylcellulose, PEG: polyethylene glycol, PPG: polypropylene glycol, SMPOE: sorbitan monolaureate poly(oxyethylene)ether, PVA: polyvinyl alchol, TB: tributyrin, TMAC: tetramethylammonium chloride, aqueous solution, W: water.

[a]Not available.

[b]μmol/(min•$g_{support}$).

[c]% w/v.

[d]μmol/ml.

[e]μmol/(min•$mg_{immobilized\ protein}$).

[f]μmol/(min•$cm^2_{support}$).

product complex (171–177). A detailed description and discussion of these more complex mechanisms will be presented in a subsequent paper.

At a molecular level, the mechanism of interesterification reactions involves hydrolysis of the ester molecule, followed by an esterification reaction. As a result, in addition to the lipase, water must be present in at least catalytic amounts (75,90). On the other hand, the presence of very large amounts of water promotes the hydrolysis reaction. Hence, one important operational consideration when one utilizes an immobilized lipase to catalyze ester synthesis and interesterification reactions is the concentration of water necessary to obtain maximum activity. Values reported in the literature are compiled in Table 8. Also included are values for hydrolysis reactions performed in a non-aqueous solvent system. It is apparent that the optimum water concentration lies in the range from 0.75 to 4% (w/v). These values are comparable in magnitude to the maximum solubility of water in commonly employed feedstocks or solvents.

IMMOBILIZED LIPASE REACTORS

The use of immobilized lipases for the modification of fats and oils is currently a subject of expanding interest. This interest is partly due to the fact that the use of lipases is more cost effective when these enzymes are employed in immobilized rather than in free form. In general, the multiphase reactors considered for this application contain a solid phase that can be either mobile or stationary (178).

Some relevant features of immobilized lipases make this approach particularly suitable for use in industrial processing of oils and fats. This technology facilitates the development of continuous, large scale commercial processes (as opposed to the small scale operations which employ soluble enzymes). The large scale systems have a high efficiency per unit volume of reactor and a corresponding high rate of return of capital costs (179). The use of immobilized lipase reactors also leads to a decrease in the potential for contamination of the product via the presence of residual lipase. It permits multiple use of the lipase with attendant consequences for process economics. It often enhances the thermal and chemical stability of the lipase (it can impart resistance to the denaturing effect of various organic solvents) and leads to predictable decay rates (180). It also enhances opportunities for better control of both the process and product quality (181).

Several reactor configurations have been used in studies of immobilized lipases. Two types of phases are invariably present—a solid phase (i.e., the carrier on which the lipase is immobilized), and one or two liquid phases (i.e., the feedstocks). If an organic solvent is used to dissolve the reactant and product species, then only a single-liquid phase is present in the reactor. The solid phase in two phase reactors and one of the liquid phases in three phase reactors may appear as a dispersed phase or as a continuous phase. A typical configuration of the continuous solid phase corresponds to a membrane in either flat sheet or hollow fiber form. Dispersed solid phases involve the use of powders as supports. Whether the organic or the aqueous phase

TABLE 8

Optimum Water Concentration for Reactions Catalyzed by Immobilized Lipases

Optimum water concentration	Type of reaction	Source of lipase	Support	Method of binding	Solvent or continuous phase	T [°C]	Reference
3.0[b]	Interesterification	*Rhizopus delemar*	Celite	Adsorption	*n*-Hexane	40	90
3.0[b]	Interesterification	*R. delemar*	ENTP	Entrapment	*n*-Hexane	40	90
0.75[a]	Hydrolysis	*R. arrhizus*	Celite	Adsorption	*n*-Hexane	36.5	118
4[b]	Ester synthesis	*Chromobacterium viscosum*	Polypropylene	Adsorption	Glycerol/OA	40	120
0.2[b]	Hydrolysis	*Rhizopus arrhizus*	Fungal mycelia	Cell binding	DIPE	50	127
11[b]	Interesterification	*Mucor miehei*	Resin	Ion exchange	Olive oil	60	132
1[b]	Ester synthesis	*Rhizopus niveus*	Dowex	Covalent	Glycerol/RBA	40	148
2.2[b]	Ester synthesis	*Pseudomonas mephitica*	Dowex	Covalent	Glycerol/RBA	60	148
1.1[b]	Ester synthesis	*Chromobacterium viscosum*	Dowex	Covalent	Glycerol/RBA	60	148
0.75[b]	Ester synthesis	*C. viscosum*	Spherosil DEA	Covalent	Glycerol/RBA	60	148
1.7[b]	Ester synthesis	*C. viscosum*	DEAEc	Covalent	Glycerol/RBA	60	148

Abbreviations: DEAEc: DEAE cellulofine, DIPE: di-isopropyl ether, ENTP: polypropylene glycol, OA: oleic acid, RBA: rice bran acid.
[a]a_w.
[b]%(w/v).

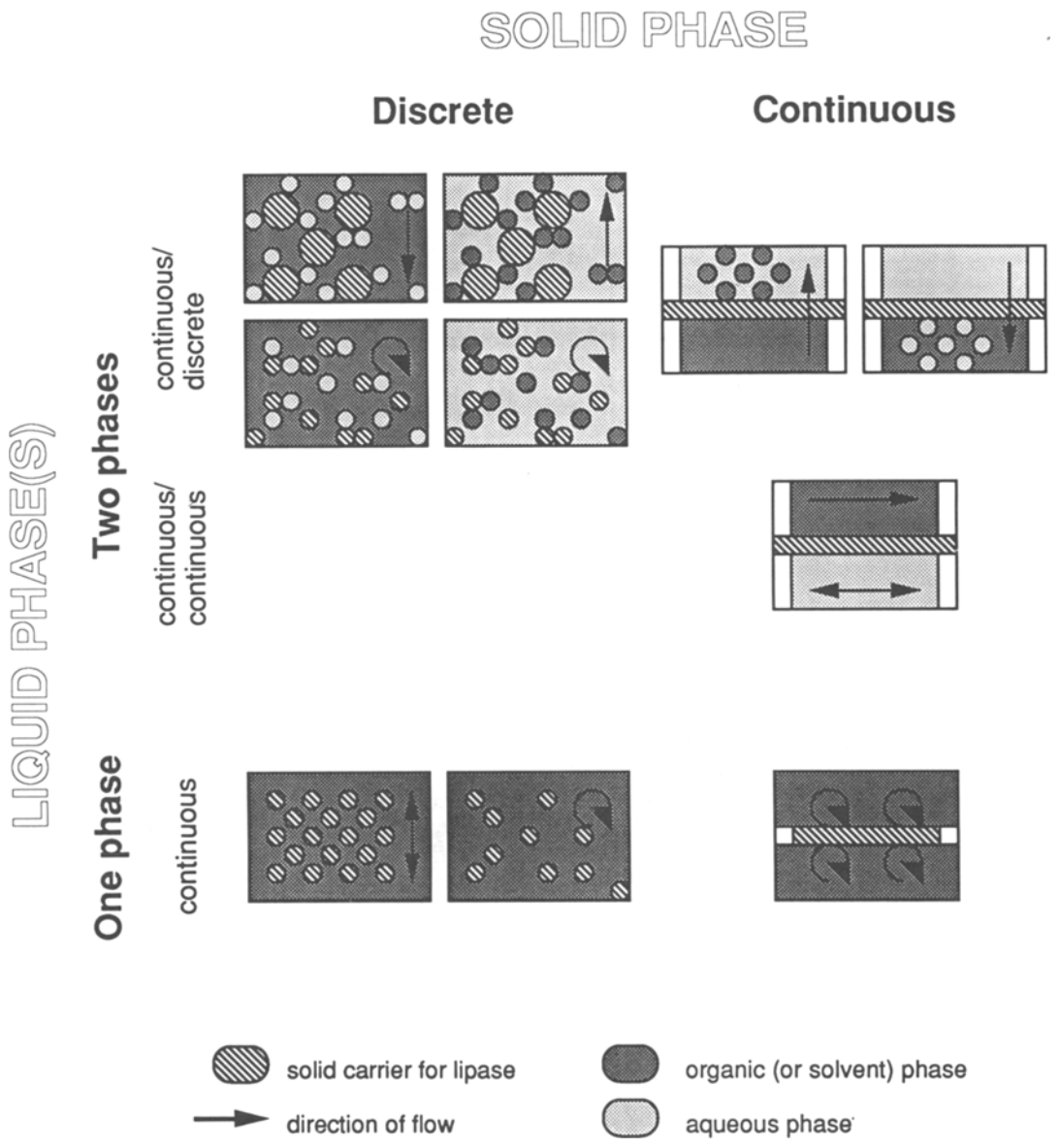

FIG. 1. Schematic representation of typical immobilized reactor configurations.

will constitute the disperse phase in three-phase reactors often depends on the relative amounts of the two liquid phases. A comprehensive classification structure of the types of immobilized lipase reactors studied to date is depicted in Figure 1. Tables 9–12 consist of a compilation of the major characteristics of the lipase reactors which have been described in the literature.

Lipases act upon substrates that are generally much more viscous than water at the same temperature. Hence, one way to overcome the difficulties associated with operating immobilized lipase reactors for processing these substrates is by the use of suitable solvents. These solvents also help to keep the water activity low. This condition is helpful for both ester synthesis and interesterification reactions. However, the single most important criterion in selecting a non-aqueous solvent is its compatibility with the maintenance of the catalytic activity (182,183) and substrate specificity (184) of the lipases. A monolayer of bound water plays a key role in maintaining the structural integrity of lipases since it affects intramolecular salt bridges and hydrophobic interactions. Hence, water-miscible solvents impart changes in conformation leading to inactivation of lipases because they extract bound water from the proteinaceous backbones of these enzymes (122,133). On the other hand, the half-lives of lipases operating in microaqueous systems are expected to be higher than those of enzymes operating in macroaqueous systems because water is required for the thermal inactivation process (185). Solvents used to carry out reactions catalyzed by immobilized lipases include benzene (122,133,149), toluene (122,133,149), *n*-hexane (90,107,117,118,122,133,134), cyclohexane (122), *n*-heptane (67,122), octane (122), isooctane (97,113,114,122), nonane (122), decane (122), hexadecane (102), isopropylether (122), diisopropylether (127,128), petroleum ether (71,109,140), tetrahydrofuran (133), triacetin (133), methylcyanide (133), acetone

(122,128), methyl isobutyl ketone (133), dimethyl formamide (133), dioxane (133), carbon tetrachloride (122), chloroform (122,149), methylene chloride (122), 1,1,1-trichloroethane (149), polyvinyl alcohol (99,101), and ethyl acetate (122). The rates of the enzymatic hydrolysis of oils by immobilized lipases are strongly affected by the polarity of the reaction solvents (122).

Membrane, or diaphragm, reactors have been employed in the presence of one (79,99,101) and two liquid phases (110–112,115,116,119,120,145). In one study involving a single liquid feedstock, a liquid crystal membrane with a lipase previously entrapped therein was fixed on a platinum cathode. This electrode and a platinum anode were immersed in a well stirred aqueous solution of sorbitan monolaureate poly(oxyethylene) ether and tetramethylammonium chloride (79) or polyvinyl alcohol (101). The molecules of the liquid crystal arranged themselves regularly under the influence of an electric field. Hence, the diffusivity of substrate through the membrane containing the liquid crystal was easily controlled. In a second type of reactor, organic and aqueous phases are separated by a solid membrane in which the lipase is immobilized. Two kinds of flow patterns have been employed: flow tangential to and flow normal to the membrane. For the tangential flow case, both phases flow parallel to the membrane in either cocurrent or countercurrent fashion (102,103,110,111,120,145). The pressure drop along the reactor coordinate is small, and no bulk flow through the membrane is allowed. For flow normal to the membrane one of the phases is pumped through the membrane, whereas the other phase remains stationary (112,115,116,119). One thus forms a coarse mixture of water and finely divided oil droplets (or *vice versa*) which is readily separated by gravity settling.

The fixed-bed reactor has traditionally been used for most large scale catalytic reactors because of its high efficiency, low cost, and ease of construction and operation. One of the prime attributes of this type of reactor is its simplicity with the attendant consequences of low costs for construction, operation, and maintenance. It requires a minimum of auxiliary equipment and is very efficient. The packed bed reactor usually provides more surface area for reaction per unit volume than does a membrane reactor. The packed bed configuration has been employed for lipase-catalyzed hydrolysis and interesterification reactions by a number of investigators (71,75,86,112,115–117,127,128). Granules of various sizes with a lipase previously attached to them (71,75,86,112,115–117) or lipase-containing dried mycelia (127,128) are usually confined in a jacketed column. If only a single liquid phase is employed, this phase may be pumped upwards in order to reduce the tendency for by-passing of fluid (117,140), or downwards in order to take advantage of the driving force of gravity (85,127,128). Solvents used in single-phase liquid systems include di-isopropyl ether (127,128), petroleum ether (71,75,140), and hexane (117). In the case of multi-phase liquid systems, the two streams may flow through the reactor in opposite directions with the more dense phase flowing downwards (112,115,116) or in the same direction (112,115,116). Variations of the basic continuous flow design include a semicontinuous mode of operation (89,100). This apparatus consists of a stirred tank connected in series with a column of water-saturated material and a packed bed reactor (89), or simply the column packed with the immobilized lipase beads (100). The outlet stream of this latter reactor is then recycled to the tank. The column of water-saturated material located prior to the packed bed reactor has the purpose of saturating the fat in the feed stream with water (130,132).

There are two operational constraints which must be considered when operating packed bed reactors: i) intraparticle diffusion limitations on reaction rates; and ii) high pressure drop across the reactor packing. Diffusional limitations can be alleviated if smaller support particles are used. However, such supports give higher pressure drops. If no solvents are used, the effect of the viscosity of the oil may be minimized only

TABLE 9

Reactors Using a Membrane Support

Type of reactor	Source of lipase	Method of binding	Support	Purpose	Reference
Submerged membrane	*Pseudomonas*	Entrapment	MBBA	Hydrolysis	79
Submerged membrane	*Mucor*	Entrapment	MBBA, CHBP	Hydrolysis	99, 101
Hollow fibers	*Candida rugosa*	Adsorption	Cellulose	Ester synthesis	102
Hollow fibers	*C. rugosa*	Adsorption	Cellulose	Hydrolysis	103
Tangential flow membrane	*C. cylindracea*	Adsorption	Polypropylene	Hydrolysis	110
Tangential flow membrane	*Rhizopus japonicus, R. delemar, Candida cylindracea, Chromobacterium viscosum, Mucor miehei, Phycomyces nitens, Pseudomonas fluorescens*	Adsorption	Polypropylene	Ester synthesis	111
Flow-thru membrane	*Candida cylindracea*	Adsorption	Polypropylene	Hydrolysis	112
Flow-thru membrane	*C. cylindracea*	Adsorption	Polypropylene	Hydrolysis	115, 116
Flow-thru membrane	*Thermomyces lanuginosus*	Adsorption	Acrylic	Hydrolysis	119
Tangential flow membrane	*Chromobacterium viscosum*	Adsorption	Polypropylene	Ester synthesis	120
Tangential flow membrane	*Aspergillus niger*	Adsorption	Polypropylene	Hydrolysis	145

Abbreviations: CHBP: 4-cyano-4′-heptylbiphenyl, MBBA: 4-methoxybenzilidene-4′-n-butylaniline.

TABLE 10

Packed Bed Reactors

Source of lipase	Method of binding	Support	Purpose	Reference
Aspergillus niger, Mucor miehei	Adsorption	Celite	Interesterification	71
Aspergillus niger, Geotrichum candidum, Rhizopus niveus	Adsorption	Kieselguhr	Interesterification	75, 139
Aspergillus niger, Pseudomonas fluorescens, Rhizopus japonica, R. delemar, Candida cylindracea, Hog pancreas	Covalent, Adsorption	Porous glass	Hydrolysis	85
Porcine pancreas, *Candida cylindracea*	Adsorption	Spherosil	Hydrolysis	86
C. cylindracea	Entrapment Adsorption Covalent	ENTP	Hydrolysis	89
Humicola langinosa	Entrapment, Cross-linking	ENTP, Amberlite Ca-alginate	Hydrolysis	100
Candida cylindracea	Adsorption	HDPE	Hydrolysis	112
C. cylindracea	Adsorption	Polypropylene	Hydrolysis	115, 116
Rhizopus arrhizus	Adsorption	Celite	Interesterification	117
R. arrhizus	Cell binding	Fungal mycelia	Hydrolysis	127
R. arrhizus	Cell binding	Fungal mycelia	Ester synthesis	128
Mucor miehei	Ion exchange	Resin	Interesterification	130
M. miehei	Ion exchange	Resin	Interesterification, Ester synthesis	132
M. miehei	Adsorption	Celite	Interesterification	140

Abbreviations: ENTP: polypropylene glycol, HDPE: high-density polyethylene.

at the expense of having to employ higher temperatures. On the other hand, higher temperatures lead to faster rates of inactivation of lipases. Economic considerations require that one achieve a compromise between these effects.

Continuous stirred tank reactors (CSTR's) possess some particular advantages over fixed bed reactors, e.g., lower construction costs and efficient stirring that eliminates the presence of concentration and/or temperature gradients. In general, however, a CSTR must be larger than a packed bed reactor to achieve the same extent of reaction. The use of a CSTR has been reported both for the hydrolysis of the triglycerides contained in olive oil in the presence of an aqueous buffer (112,115,116) and for the synthesis of glycerides from fatty acids and glycerol dissolved in di-isopropyl ether (128). In order to prevent the immobilized lipase from leaving the CSTR, a microfilter (128) or a screen (112,115,116) must be provided at the reactor outlet.

Fluidized bed reactors also have been employed to effect the hydrolysis of glycerides. For example, a fluidized bed reactor employing a recycle stream has been used with an emulsion of tributyrin in water (84). This type of reactor has several advantages over a fixed bed reactor, namely, lower pressure drop at high flow rates, less channeling, reduced coalescence of the emulsion particles, freedom from plugging by feed particulates, and ease of simulation due to the absence of concentration gradients.

The stirred batch reactor is the type of reactor most commonly employed in bench scale and industrial scale applications. Batch reactors are extremely versatile and easy to operate. Configurations include glass flasks stirred with magnetic bars (67,71,75,89,91–94, 96–98, 100, 106, 109, 123, 124, 126, 129, 131, 133, 134, 138,139) and vessels stirred by submerged impellers (140), as well as flasks mounted in reciprocal oscillators (85,90,95,121,148) or end-over-end rotators (83,118). The mixing pattern avoids temperature and concentration gradients. In the case of the well-stirred batch reactor, sampling can be accomplished at a single, arbitrarily located point. In emulsions, the most common feedstock configuration for hydrolysis, lipases immobilized on powdered supports are very likely to collide with suspended oil droplets.

For interesterification reactions, stirred batch reactors in which the lipase is immobilized on powdered supports have been used extensively. Lipase-catalyzed hydrolysis reactions, which proceed very slowly when compared with other enzyme-catalyzed reactions, are roughly one order of magnitude faster than interesterification reactions; continuous flow reactors would, thus, require extremely large reactor volumes and very small flow rates for reasonable degrees of interesterification. Both these constraints render continuous flow stirred tank reactors impractical on a bench scale. Stirred batch reactors, on the other hand, do not require pumping devices. Furthermore, stirred batch reactors can be made very small because sampling is made along a virtually unbound time coordinate rather than along a necessarily limited spatial coordinate.

Since stirred batch reactors can be made very small, heating and cooling operations proceed extremely fast. To achieve measurable rates of interesterification, it

TABLE 11

Stirred Batch Reactors

Source of lipase	Method of binding	Support	Purpose	Reference
Geotricum candidum, Aspergillus, Mucor miehei, Alcaligenes, Pseudomonas, hog pancreas, *Candida cylindracea, Rhizopus arrhizus, R. delemar*	Adsorption, entrapment	Spherosil, porous glass, alumina, titania, Celite, polyurethane	Ester synthesis	67
Aspergillus niger, Mucor miehei	Adsorption	Celite	Interesterification	71
Aspergillus niger, Candida cylindracea, Geotrichum candidum	Adsorption	Kieselghur	Interesterification	75, 139
Porcine pancreas	Covalent	Sephadex, Sepharose, cellulose	Hydrolysis	83
Aspergillus niger, Pseudomonas fluorescens, Rhizopus japonica, R. delemar, Candida cylindracea, Hog pancreas	Covalent, adsorption	Porous glass, Sepharose, Dowex	Hydrolysis	85
Porcine lipase	Covalent	Sepharose	Hydrolysis	87, 88
Candida cylindracea	Entrapment, adsorption, covalent	Duolite, Celite, CPG, Spherosil, ENT, ENTP, polyurethane	Hydrolysis	89
Rhizopus delemar	Entrapment, adsorption	Celite, polyurethane, Spherosil, ENT, ENTP,	Interesterification	90
Microbial	Covalent	Agarose	Hydrolysis	91, 92
Candida cylindracea	Covalent, entrapment	Sepharose polyacrylamide	Hydrolysis	93
Cotton seed, Pancreas	Covalent	Polyamide	Hydrolysis	94
Mucor javanicus	Covalent, ion exchange	AEC, agarose, DEAE-cellulose, QAE-Sephadex	Hydrolysis	95
n.a.[a]	Covalent, ion exchange	PAS, CIE	Hydrolysis	96
Candida rugosa, Rhizopus arrhizus	Entrapment	Polyethylene glycol, polypropylene glycol	Hydrolysis, interesterification	97
R. delemar	Entrapment	AA, ACa, ANa, HEMA, divinyl sulfone, P	Hydrolysis	98
Humicola lanuginosa	Entrapment, adsorption, covalent, cross-linking	ENTP, polyurethane, Ca-alginate, ADCPG Amberlite, Diaion, octyl-Sepharose	Hydrolysis	100
Aspergillus oryzae, A. niger, animal pancreas	Microencapsulation	Butterfat	Hydrolysis	104
porcine pancreas, *Chromobacterium viscosum*	Microencapsulation	Silica, dextran	Hydrolysis	105, 142, 143
Porcine pancreas	Adsorption	Glass	Hydrolysis	106
Oilseed rape	Adsorption	Celite	Ester synthesis	107
cotton seed	n.a.	n.a.	Interesterification	108
Aspergillus	Adsorption	Celite, Filtercel Hyflo supercel, CPS	Interesterification	109, 138
Candida rugosa	Adsorption	Sephadex	Hydrolysis	113, 114
Rhizopus arrhizus	Adsorption	Celite	Interesterification	118
Chromobacterium viscosum	Adsorption	Palmitoyl cellulose	Hydrolysis	121
Candida rugosa	Adsorption	Sephadex	Hydrolysis	122
C. cylindracea	Adsorption	Sepharose, octyl-Sepharose, phenyl-Sepharose	Hydrolysis	123
C. cylindracea	Adsorption	Kaolin, silica gel, activated carbon, avicel	Hydrolysis	124
Pseudomonas mephitica	Adsorption	DADA	Hydrolysis	125
P. mephitica, Candida cylindracea, pancreas	Adsorption	BTAS, HXAS, HPAS, OTAS, NLAS, DCAS, UDAS, DDAS, TDAS	Hydrolysis	126

(Continued)

TABLE 11 (Continued)

Source of lipase	Method of binding	Support	Purpose	Reference
Rhizopus arrhizus	Cell binding	Fungal mycelia	Hydrolysis	127
Pseudomonas mephitica	Cell binding	Bacterial cell debris	Hydrolysis	129
Mucor miehei	Ion exchange	Synthetic resin	Hydrolysis	131
M. miehei	Ion exchange	Synthetic resin	Ester synthesis	133
M. miehei	Ion exchange	Synthetic resin	Interesterification	134
M. miehei	Ion exchange	Synthetic resin	Ester synthesis	135, 136
Geotrichum candidum	Adsorption, covalent	CMC, PAB, CMS, CA, Sepharose, enzacryl, aminocaproin, PPAS, Wofatit, Servachrom, kieselguhr, alumina, CPG	Hydrolysis	137
Mucor miehei	Adsorption	Celite	Interesterification	140
Rhizopus microsporus	Adsorption	AE-cellulose	Hydrolysis	141
R. oryzae	Covalent	Alumina	Hydrolysis	144
Pseudomonas mephitica, P. fluorescens, P. cepacia, Mucor miehei, M. javanicus, Candida cylindracea, Rhizopus niveus, Aspergillus niger, Geotrichum candidum, Chromobacterium viscosum, hog pancreas, wheat germ	Cross linking	Dowex, Spherosil, DEAE cellulofine	Ester synthesis, interesterification	148
Pseudomonas fragi	Covalent	Magnetite	Ester synthesis	149

Abbreviations: AA: 23% acrylamide+1.3%NN'methylene *bis* acrylamide (aq), ACa: 30% calcium acrylate (aq), ADCPG: alkylamine derivative of controlled pore glass, AEC: aminoethylcellulose, ANa: 30% sodium acrylate (aq), BTAS: butylamine-Sepharose, CA: carboxylagarose, CIE: carboxylic ion exchange resin, CMC: carboxymethylcellulose, CMS: carboxymethyl-Sephadex, CPG: controlled pore glass, DADA: 1,12-diamino-dodecane agarose, DCAS: decylamine-Sepharose, DDAS: dodecylamine-Sepharose, ENT: polyethylene glycol, ENTP: polypropylene glycol, HEMA: 30% hydroxyethylmethacrylate (aq), HPAS: heptylamine-Sepharose, HXAS: hexylamine-Sepharose, NLAS: nonylamine-Sepharose, OTAS: octylamine-Sepharose, P: 20% 1-vinyl-2-pyrrolidone (aq), PAB: *p*-aminobenzylcellulose, PAS: polyamino-polystyrene resin, PPAS: poly-*p*-aminostyrol, TDAS: tridecylamine-Sepharose, UDAS: undecylamine-Sepharose.
[a]Not available.

TABLE 12

Stirred Continuous Reactors Using an Immobilized Lipase

Type of reactor	Source of lipase	Method of binding	Support	Purpose	Reference
Fluidized bed	Pancreas	Covalent, crosslinking	Stainless steel, polyacrylamide	Hydrolysis	84
CSTR	*Candida cylindracea*	Adsorption	Celite, cellulose, ethyl cellulose, silica gel, kieselguhr, clay, alumina, CPG, carbon, nylon, HDPE, polypropylene	Hydrolysis	112, 115, 116
CSTR	*Rhizopus arrhizus*	Cell binding	Fungal mycelia	Ester synthesis	128

Abbreviations: CPG: controlled pore glass, CSTR: continuous flow stirred tank reactor, HDPE: high-density polyethylene.

is usually necessary to activate the catalyst particles by hydration with up to 10% of their weight of water (186). The addition of solvents aimed at quenching the lipase-catalyzed reaction is also easy. After the batch reaction is terminated, separation of the powdered lipase support from the reaction fluid can be accomplished by simple filtration or centrifugation. When colloidal magnetic particles are employed as a support for lipase, separation of the immobilized lipase from the reaction mixture may be carried out with an electromagnet (149).

PROCESS CONSIDERATIONS

When assessing the potential application of a new technology in a manufacturing plant, one must address two questions: i) whether the process can produce the desired end product in good yield and at the desired degree of purity (technical feasibility); and ii) whether the process can produce the product at a reasonable cost (commercial feasibility) (130). The former can be demonstrated in small systems on a bench scale, whereas assessment of the latter question deals with economic considerations involving both capital costs and operating costs as well as the market for the product(s).

TABLE 13

Productivities of Reactors Employing Immobilized Lipases Over a Time Period Equal to Two Half-Lives

Productivity ($kg_{product}$/ $kg_{immobilized\ lipase}$)	Type of reactor	Type of reaction	T (°C)	pH	Reference
100	Fixed bed	Hydrolysis	40	7	112
1700	Membrane	Hydrolysis	n.a.[a]	7	112
1100	CSTR	Hydrolysis	35	7	112
104	Fixed bed	Hydrolysis	40	7	115, 116
1695	Membrane	Hydrolysis	n.a.	7	115, 116
308	CSTR	Hydrolysis	35	7	115, 116
3500	Packed bed	Ester synthesis	n.a.	n.a.	132
5000	Packed bed	Interesterification	n.a.	n.a.	132
1200	Stirred batch	Ester synthesis	60	n.a.	133

[a]Not available.

As time on stream elapses, immobilized lipases are known to lose activity due to thermal deactivation. Hence, one important operating consideration associated with the long-term use of lipase in an industrial bioreactor is how to deal with its thermal deactivation. The economic feasibility of the industrial process may hinge on the useful lifetime of the enzyme (81). It has been suggested that the operating temperature of an immobilized lipase reactor be increased at a rate which compensates for decreases in enzymatic activity, thereby permitting the system to maintain constant conversion and throughput (187). Although increasing the temperature will increase the reaction rate, higher temperatures also accelerate the rate of enzyme denaturation (extreme increases in temperature will completely and irreversibly denature lipases).

Sometimes the operating temperature must be held constant due to processing constraints (e.g., minimization of oxidation of fats while in the reactor), because the lipase will operate effectively only within a narrow temperature range, or because the amount of lipase adsorbed will decrease following a temperature increase (150). If the temperature must be held constant, then either constant flow rate can be maintained (at the concomitant expense of a reduction in extent of conversion as time proceeds), or constant conversion can be maintained by reducing the flow rate (130), thereby increasing the average residence time of the substrate in the reactor. Shortcomings of these procedures are steady decreases in productivity (amount of product produced per unit amount of catalyst) with time. The specific strategy chosen to compensate for lipase deactivation depends on process constraints. Several productivities reported for immobilized lipase reactors are listed in Table 13. Fixed bed reactors show low productivities for hydrolysis, but enhanced productivities for reactions taking place in microaqueous systems. Membrane reactors possess very high productivities, especially if a tangential flow pattern is employed.

Mechanical forces can disturb the complex shape of a lipase molecule to such a degree that deactivation can occur. Examples of such detrimental mechanical forces include shear effects created when i) fluids flow in long, densely packed bed reactors; ii) oil is forced through pores of submicroscopic size in flow-thru membrane reactors; and iii) fast stirring of the oil/water dispersion in perfectly stirred or fluidized bed reactors is employed (81). Lipase from *Candida cylindracea* has been reported to lose activity as a function of shearing time and shear rate in stirred tank reactors (188). In the presence of polypropylene glycol, the rate of denaturation of the lipase decreased by 93% (188). Below 30°C, temperature was found to have no effect on the rate of shear-induced denaturation of the lipase, but the rate of denaturation increased dramatically above 30°C (188).

Several methods exist for improving lipase stability. These methods may involve genetic engineering (use of improved microbial strains); chemical modification; and physical treatment.

Researchers are trying to identify lipases which are intrinsically more stable and capable of producing more selective release of free fatty acids. Examples include studies of a strain of *Aspergillus niger* that produces lipases which are very specific towards short-chain fatty acid residues (189), and of a strain of *A. oryzae* which is able to produce cheddar-type flavors (190). Recombinant DNA techniques have also augmented the potential for new developments in the area of lipases, e.g., the commercial production of accelerated cheese ripening systems (191). Fundamental studies of the production capacity of multiform lipases by a number of fungi have interested some investigators (192). The production mechanisms include those for which the lipases are coded by entirely different genes, and those for which a single lipase protein is biosynthetized and subsequently modified so that it behaves differently from the parent protein. In the latter case, modification of the parent protein to produce lipases with different characteristics can arise from: i) interactions with various kinds of lipids (193); ii) partial degradation effected by a coexisting protease (194); or iii) association/dissociation equilibrium of subunits in different ways (195).

Chemical treatments commonly used with lipases can involve modification of the proteinaceous backbone with polyethylene glycol in order to render the enzyme very active in organic solvents (149,196-209). This procedure preserves the shell of hydration around the lipase and keeps the enzyme physically separated from the external organic environment. This approach creates a microheterogeneous environment which is characterized by a very high interfacial area (180).

Solvents comprised of polyalcohols diminish the tendency for rupture of the hydrogen bonds that play an important role in maintaining the tertiary structure

of the lipase (210). Stabilization of lipase by glycerol has been reported (110,111,120). Calcium cations have been implicated in stabilization of the tertiary structure of several lipases. By forming ionic bonds with two different amino acid residues, Ca^{2+} can serve as a stabilizing bridge (81). Calcium salts are efficient stabilizers and even activators of immobilized lipases (120). However, formation of insoluble calcium soaps inside the process reactor constitutes a major drawback, especially if flow-through patterns are employed (119).

The degree of purity of the feedstocks to be processed plays a very important role in determining the operating life of the immobilized lipase. Refining, bleaching, and deodorizing operations may introduce traces of substances that poison the lipase (130). Free heavy metal cations can be removed via use of chelating agents (e.g., citric acid or EDTA) added to the buffer solution. Unsaturated oils are also very susceptible to oxidation. In the case of edible oils and fat-containing foods, long residence times, high temperatures, and the presence of oxygen or free radicals inside the reactor may lead to the development of various off-flavors and off-odors. These factors make the food unacceptable and reduce its shelf-life (2). The extent of detrimental changes arising from oxidation may be reduced by using suitable antioxidants (e.g., citric acid).

The benefits of a solvent-free system over a solvent-based system should be balanced against its disadvantages. Solvent-free systems offer several advantages, including greater safety, reduction in solvent extraction costs, increase in reactant concentration in the reactor and consequent reduction in mass transfer limitations (140). These factors must be weighed against the effects on the catalyst activity caused by the increased solubility of water in the reactants and the increased pumping costs associated with use of the viscous reactant mixture (140).

Some of the major obstacles to the use of immobilized enzyme bioreactors within the oleochemistry industry arise because the number of high added value products in the oleochemical industry is limited, the enzymes involved in the biotransformations of the lipids are costly; and there are many difficulties in solving engineering problems because of heterogeneous and/or microaqueous nature of some biochemical reactions (1,211). At present, lipases represent no more than 3% of all enzymes used in industry (212). However, increased costs of energy obtained from fossil fuels and increased demand for higher quality products coupled with vary narrow purity specifications are likely to lead to new incentives for the biochemical transformation of fats and oils. Hence, immobilized lipase processes offer great potential for future development.

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