LIPASE-CATALYZED BIOCHEMICAL REACTIONS IN NOVEL MEDIA: A REVIEW

MAYANK T. PATEL*, R. NAGARAJAN** and ARUN KILARA***

The Pennsylvania State University, University Park, PA 16802

(Received September 15, 1995; in final form January 11, 1996)

Lipids in biological matter are mostly triacylglycerols (TAG). Lipolytic enzymes, primarily lipases, are indispensable for bioconversion of such lipids from one organism to another and within the organisms. In addition to their biological significance, lipases are very important in the field of food technology, nutritional and pharmaceutical sciences, chemical and detergent industries, and clinical medicine because of their ability to catalyze various reactions involving a wide range of substrates. Conventionally, lipases have been viewed as the biocatalysts for the hydrolysis of TAG (fats and oils) to free fatty acids, monoacylglycerols (MAG), diacylglycerols (DAG), and glycerol. The main advantages of lipase catalysis are selectivity, stereospecificity, and mild reaction conditions. Despite these advantages and the fact that enzymatic splitting of fats for fatty acid production was described as early as in 1902, the lipase-catalyzed process has not replaced the commercial physicochemical process for the continuous splitting of TAG utilizing super-heated steam. The limited exploitation of lipase technology may be attributed to high enzyme cost, large reaction volume, requirement for emulsification of substrate, and risk of microbial contamination. Many of these limitations originate from the fact that lipases are employed mainly in water-rich reaction media where the solubility of the substrate TAG is very small. To circumvent this problem and to realize the full potential of lipase, researchers have explored newer approaches by manipulating the conditions under which the lipases act. Many of these novel approaches for lipase catalysis have been the outcome of the discovery that enzymes can be active in water-poor, non-polar media (Hanhan, 1952; Misiorowski and Wells, 1974; Zaks and Klibanov, 1984). Also, the finding that lipases can act in organic solvents has led to an expansion of their applicability in a wide variety of chemical reactions. Lipase catalysis in some of the well established reaction media has previously been reviewed (Brockerhoff and Jensen, 1974; Brockman, 1984; Lilly et al., 1987; Halling, 1990; Inada et al., 1990; Malcata et al., 1990). The present review is intended to present a compilation and comparison of novel reaction systems used for lipase catalysis. This review describes briefly the general characteristics of lipase reactions, applications of lipase in various fields, and conventional lipase technology. The lipase-mediated biochemical reactions, particularly the hydrolysis of TAG in novel reaction media is discussed in greater detail.

KEYWORDS Lipase catalysis Enzymes in organic media Hydrolysis of triacylglycerols Lipase applications Lipase in novel media Lipase-catalyzed reactions

I. GENERAL CHARACTERISTICS OF LIPASE CATALYSIS

Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) can be viewed as a special class of esterases. Esterases preferentially catalyze hydrolysis reactions of soluble esters whereas lipases are distinguished by their ability to catalyze the hydrolysis of insoluble long-chain fatty acid esters. Although lipases do exhibit some activity toward
soluble substrates, the rate of hydrolysis is generally slow compared to the rate of hydrolysis of triacylglycerol emulsions. The natural substrates of lipase are triacylglycerols of long-chain fatty acids (fats and oils) which on hydrolysis yield diacylglycerols, monoacylglycerols and, ultimately, glycerol, with fatty acids being released at each step (Fig. 1). Depending upon the origin of lipases, the fatty acids are released either randomly from any position or preferentially from a specific position.

**Interfacial Catalysis**

Interfaces provided by either aggregated or dispersed substrate in the aqueous medium constitute the primary site for lipase catalysis. Lipase hydrolyzes triacylglycerols at the interface between the insoluble substrate phase and the aqueous phase, in which the enzyme is solubilized (Entressangles and Desnuelle, 1968; Brockman et al., 1988). The presence of aggregated substrates providing an interface for the reaction has been demonstrated in experiments involving water-soluble substrates such as triacetin and tripropionin (Entressangles and Desnuelle, 1968). It was shown that, for a lipase but not for an esterase, a stepwise increase in activity occurred only when the solubility limit of the monomeric substrates was surpassed, i.e., above the critical micellar concentration of the substrate.

The hydrolysis of triacylglycerols by lipase occurs in a heterogeneous system comprised of multiple phases. In such systems, the substrate, products, and enzyme partition themselves among the bulk and surface phases. This distribution is not fixed but changes as lipolysis proceeds (Patton and Carey, 1979; Brockman, 1984). These features lead to difficulties in the kinetic analysis of the reaction using enzyme kinetic models developed for reactions in homogeneous aqueous systems (in which both the enzyme and substrate are soluble). Benzonana and Desnuelle (1965) have pointed out the importance of interfacial area rather than the bulk concentration of substrate and have shown that lipolysis will apparently conform to the Michaelis-Menten equation when the substrate concentration is expressed on the basis of interfacial area rather than the bulk system volume.

![FIGURE 1 Lipase catalyzed hydrolysis of triacylglycerol to glycerol and fatty acids. R1, R2 and R3 represent fatty acids at the sn-1, sn-2 and sn-3 position, respectively, on the glycerol moiety.](image-url)
The lipase reaction can be envisaged as a two-step process: adsorption of the enzyme at interfaces and catalysis by the adsorbed enzyme. The first step which precedes the formation of enzyme-substrate complex is the adsorption of lipase onto the surface of substrate phase in a non-specific manner. The second step of the catalytic reaction proceeds at the interface and, ultimately, the enzyme is regenerated with the liberation of the product. It has been proposed that adsorption of lipase at the interface could occur independently of the catalysis in the interfacial region (Brockman et al., 1973; Momsen and Brockman, 1981; Brockman, 1984; Sugiura, 1984). This implies that lipases possess a molecular structure capable of binding to the substrate-containing interface at a site that is functionally distinct from the catalytic site of the enzyme. Through this binding, presumably, the active site is localized and oriented in proximity to the ester bonds of triacylglycerol and the catalytic reaction thus occurs. Brockman et al. (1973) suggested that the simple partitioning of a lipase and its substrate between the bulk phase and lipid-water interface can stimulate lipolysis by increasing their concentration in the surface phase. It has been proposed that adsorption may also change the intrinsic catalytic activity of the enzyme, either as an immediate consequence of adsorption, or as a slow, post-adsorptive conformation change (Verger, 1980; Brockman, 1984). One could speculate that if lipase catalysis should occur at the interface, the enzyme should have greater surface activity compared to other enzymes which act in bulk phases. This is confirmed by the fact that the surface tensions of 0.001 weight percent solutions of various lipases are in the range 56–66 dyne/cm compared to 69–73 dynes/cm obtained for other enzyme solutions (Sugiura, 1984).

Recent interest in lipase catalysis in organic solvents or in non-polar environments (Butler, 1979; Fukui and Tanaka, 1982; Klibanov, 1986, 1989; Slater, 1988; Yamane et al., 1988; Andersson and Hahn-Hagerdal, 1990) demands a better understanding of the interfacial nature of lipolysis, given the absence of a bulk aqueous phase and the importance of a true interface in the majority of such reaction systems. Expression and maintenance of catalytic activity by lipase in non-polar systems may be attributed to possible conformational changes analogous to the "interfacial activation" through change in conformation occurring at interfaces in aqueous systems.

**Reversibility of Lipase Reactions**

The natural function of lipases is to catalyze the hydrolysis of TAG. However, this reaction is reversible and these enzymes, under appropriate conditions, can catalyze the formation of TAG and partial acylglycerols from glycerol and free fatty acids. The water content of the hydrolytic reaction system is a crucial factor that controls the equilibrium between the forward and reverse reactions. The reaction in reverse direction during hydrolysis also depends upon the enzyme source (Okumura et al., 1981). The reversible nature of the lipase reaction not only offers the possibility of formation of esters (esterification reaction), but also offers the possibility of transfer or exchange of the acyl moiety (interesterification) from one molecule to another. Figure 2 schematically depicts various types of reactions catalyzed by lipases. In
addition to these reactions, lipases can also catalyze thiotransferase (Zaks and Klibanov, 1985; Nagao and Kito, 1990), aminolysis (Kirchner et al., 1985; Zaks and Klibanov, 1985), and oligomerization (Abramowicz and Keese, 1989; Geresh and Gilboa, 1990) under suitable conditions.

Lipase Distribution and Specificities

Lipases are widely distributed in various animals, microorganisms, and plants (Brockerhoff and Jensen, 1974; Shahani, 1975; Brockman, 1984). Lipases from different sources may have different properties in terms of substrate specificity, thermal and pH stability, and optimum conditions for activity, such as pH, temperature, substrate concentration and ionic strength. Characteristics of various lipases from animals (Brockerhoff and Jensen, 1974; Shahani, 1975; Hamosh, 1984; Olivecrona and Bengtsson, 1984; Verger, 1984), plants (Brockerhoff and Jensen, 1974; Shahani, 1975; Huang, 1984), and microorganisms (Brockerhoff and Jensen, 1974; Shahani, 1975; Macrae, 1983a; Iwai and Tsujisaka, 1984; Sugiura, 1984) have been comprehensively reviewed.
Examples of practical importance are porcine pancreatic and ruminant lingual (pregastric) lipases (animal lipases); castor bean \textit{(Ricinus communis)}), rape seed \textit{(Brassica napus)}, and oat caryopses \textit{(Avena sativa)} lipases (plant lipases); and \textit{Chromobacterium viscosum}, \textit{Pseudomonas fluorescens}, \textit{P. fragi}, \textit{Humicola lanuginosa}, \textit{Aspergillus niger}, \textit{Rhizopus delemar}, \textit{R. arrhizus}, \textit{R. javanicus}, \textit{Geotrichum candidum}, \textit{Mucor miehei}, \textit{Penicillium cyclopium}, and \textit{Candida cylindracea} lipases (microbial lipases).

Generally, lipases from various sources exhibit differences in substrate specificities. The substrate specificity of a lipase can be related to its positional or regio-selectivity, i.e., ability to hydrolyze or synthesize ester bonds at only \textit{sn-1(3)} or both \textit{sn-1(3)} and \textit{sn-2} positions; to its fatty acid selectivity, i.e., preference for particular chain length, level of unsaturation or structure of fatty acid; to its stereo-selectivity, i.e., ability to act on either the \textit{R}- or \textit{S}-isomer of asymmetric compounds, and for triacylglycerol, the (hypothetical) ability to hydrolyze ester bonds at only the \textit{sn-1} or \textit{sn-3} position. A few examples of such lipase selectivities are listed in Table 1.

Specificity of lipases seems to be related to their origin and also to the isolation and purification process. For a lipase acting in a heterogeneous system, substrate specificity may depend on both the chemical structure of substrate and the physical properties of interface or emulsion. Although most studies emphasize the chemical specificity of lipases (Yamaguchi \textit{et al.}, 1973; Sugiura \textit{et al.}, 1974; Umemoto and Sato, 1978; Chander \textit{et al.}, 1979; Adams and Brawley, 1981), there is evidence suggesting that physical factors such as reaction temperature and surface pressure can control specificity (Sugiura, 1984). With increasing research efforts in the area of lipase catalysis in non-aqueous media, newer or altered specificities of the lipases may be discovered.

\textit{Mechanism of Lipase Action}

Little effort has been expended on the elucidation of the nature of active site and the exact mode of lipase action. Investigation on \textit{Pseudomonas} and \textit{Chromobacterium} lipases by chemical modification techniques suggested that histidine may be involved in the catalytic action (Sugiura, 1984). Pancreatic lipase is reported to be a \textit{‘serinehistidine’} enzyme (Brockman \textit{et al.}, 1973; Brockerhoff and Jensen, 1974; Shahani, 1975). This observation is based on the photooxidative inactivation of the lipase and inhibition of the lipase by high concentration of diisopropyl fluorophosphate (DFP) and diethyl-p-nitrophenyl phosphate.

Recently, Brady \textit{et al.} (1990) reported the three-dimensional structure of \textit{Mucor miehei} lipase. The x-ray structure revealed that a trypsin-like catalytic triad forms the catalytic center of this lipase. The triad is close to the surface, buried under the head of a long chain folded onto the triad. The residues forming the catalytic triad in lipase are Ser 144, His 257, and Asp 203. It is proposed that the lipase may be working in two stages: the loop is removed or displaced, possibly through interfacial activation, then the ester bond is subsequently hydrolyzed by an acid-base mechanism very similar to that for serine proteases. The loop may also serve as a device to inhibit the proteolytic activity of the triad, thereby protecting the lipase itself.
<table>
<thead>
<tr>
<th>Selectivity</th>
<th>Preference, Specificity or Discrimination</th>
<th>Enzyme Source (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1] Regio-selectivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-specific</td>
<td>No specificity</td>
<td>Candida cylindracea Sonnet, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Geotrichum candidum Tahoun, 1987; Sonnet, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chromobacterium viscosum Sugiuira and Isobe, 1975</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penicillum cyclopium Iwai and Tsujisaka, 1984</td>
</tr>
<tr>
<td>Position-specific</td>
<td>Preference for sn-1 and sn-3 over sn-2</td>
<td>Pancreatic lipase Brockerhoff and Jensen, 1974; Verger, 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aspergillus niger Okunuma et al., 1976; Iwai and Tsujisaka, 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rhizopus delemar Okomura et al., 1976; Iwai and Tsujisaka, 1984</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[2] Fatty acid selectivity</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chain length</td>
<td>Preference for short chain</td>
<td>Penicillum caseicolum Alhir et al., 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penicillum cyclopium Okunuma et al., 1976; Iwai and Tsujisaka, 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pseudomonas fragi Nishio et al., 1987b</td>
</tr>
<tr>
<td></td>
<td>Preference for medium chain</td>
<td>Rhizopus arrhizus Sonnet, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mucor miehei Sonnet, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aspergillus niger Iwai and Tsujisaka, 1984; Sonnet, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chromobacterium lipase B Sugiuira and Isobe, 1975</td>
</tr>
<tr>
<td></td>
<td>Preference for long chain</td>
<td>Geotrichum candidum Iwai and Tsujisaka, 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Candida cylindracea Sonnet, 1988</td>
</tr>
<tr>
<td>Level of saturation</td>
<td>Preference for saturated</td>
<td>Fusarium oxysporum f. Kwon et al., 1987a</td>
</tr>
<tr>
<td></td>
<td>Preference for unsaturated</td>
<td>Chromobacterium lipase B Sugiuira and Isobe, 1975; Sugiuira, 1984</td>
</tr>
<tr>
<td></td>
<td>Prefer</td>
<td>Rhodotorula pilimanae Muderhwa et al., 1986</td>
</tr>
<tr>
<td></td>
<td>C18:3 &gt; C18:2 &gt; C18:1 &gt; C18:0</td>
<td></td>
</tr>
<tr>
<td>Specificity for C18 ω-9 cis</td>
<td>Geotrichum candidum Tahoun, 1987</td>
<td></td>
</tr>
</tbody>
</table>
LIPASE CATALYZED REACTIONS

TABLE I (Continued)

<table>
<thead>
<tr>
<th>Fatty acid structure</th>
<th>Specificity for Trans-6 over Cis-6</th>
<th>Rape seed (Brassica napus)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Discriminates against Cis-4 and Cis-6</td>
<td>Rape seed (Brassica napus)</td>
</tr>
</tbody>
</table>

[3] Stereo-selectivity

<table>
<thead>
<tr>
<th>Enantio-selectivity</th>
<th>Specificity for R-alcohols</th>
<th>Mucor miehei Sonnet, 1988</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pseudomonas fragi Nishio et al., 1989</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specificity for R-esters</th>
<th>Pseudomonas fluorescens Xie et al., 1987, 1988</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Candida cylindracea Xie et al., 1988</td>
</tr>
</tbody>
</table>

| Specificity for S-ester | Pseudomonas aeruginosa Hamaguchi et al., 1986 |

II. APPLICATIONS OF LIPASE

Various lipases, alone or in combination with other enzymes, have been used in dairy and other food processes, and lipases produced in situ by microorganisms are important in making foods palatable and acceptable. Manufacturers of lipases have suggested that the enzymes may be used in detergents, leather processing, pharmaceuticals, cosmetics, etc. Industrial applications of lipases in those fields have been previously reviewed (Posorske, 1984; Rattray, 1984; Kilara, 1985; Nielsen, 1985; Welsh et al., 1989; Mukherjee, 1990; Nagao and Kito, 1990).

Current focus on the use of lipases is for the processing of fats and oils; fatty acid production, glyceride synthesis, and interesterification. Standard technology for fatty acid production involves high-temperature and high-pressure counter-current steam splitting (Sonntag, 1988). This reaction can also be achieved enzymatically and has been the subject of many publications (Linfield et al., 1984b; Hoq et al., 1985b; Tahoun et al., 1987; Buhler and Wandrey, 1988; Hirano, 1988). The rate and degree of hydrolysis varies with the reaction conditions, but under certain conditions palm oil could be completely hydrolyzed within 3 hours (Khor et al., 1986).

From a commercial viewpoint, lipase-catalyzed hydrolysis of fats for the production of fatty acids and glycerol appears to be less economical than conventional fat splitting, mainly because of the relatively high cost of lipase preparations (Buhler and Wandrey, 1987). However, if one considers the production of specific products of high commercial value, such as polyunsaturated (ω-3) fatty acids by hydrolysis of marine oils catalyzed by non-specific lipases, such processes could be economically attractive. Polyunsaturated (ω-3) fatty acids, which are important as dietetic products (Lawson and Hughes, 1988), cannot be obtained by conventional steam splitting without substantial decomposition. Similarly, γ-linolenic acid, a useful dietary constituent of certain seed oils, such as evening primrose oil (Mukherjee and
Kiewitt, 1987), can be prepared as a concentrate together with linoleic acid by lipase-catalyzed hydrolysis under mild conditions (Hills et al., 1989). Furthermore, very-long-chain monounsaturated fatty acids, such as gadoleic, erucic and nervonic acids, which are of interest to the oleochemical industry (Princen and Rothfus, 1984), can be obtained by partial hydrolysis of some seed oils, such as mustard (Sinapis alba), using sn-1,3-specific lipases. Partial hydrolysis of oils catalyzed by lipases for the production of monoacylglycerols (Holmberg and Osterberg, 1988), that are useful as emulsifiers, is another possible application of enzymatic hydrolysis of fats for the preparation of products of reasonable commercial value.

A second area of potential application of lipases is the reversal of the hydrolysis reaction (Hoq et al., 1984; Hills et al., 1989) to synthesize glycerides. By decreasing the amount of water, it is possible to shift the equilibrium position toward the synthesis. Using lipases from A. niger and C. rugosa under low water conditions and an excess of fatty acids, various acylglycerols have been synthesized. The type of products obtained could be controlled by using enzymes with regiospecificity. If lipases derived from R. delemar or A. niger were used, only mono- and diacylglycerides were formed; triacylglycerides could not be synthesized under these conditions (Tsujisaka et al., 1977). In general, the esterification process proceeds slowly but has the advantage of taking place under mild conditions and without having to resort to the use of fatty acyl halides.

Lipase-catalyzed reactions can be employed for the "deacidification" of fats and oils by esterifying the undesirable fatty acids that are present in unrefined oils. The esterification can produce either di- and monoacylglycerols that also occur in such oils or glycerol and acylglycerols. This is an alternative approach to conventional "fat refining" by alkali neutralization or distillative deacidification (Schuch and Mukherjee, 1989). The commercial immobilized lipase preparation from Mucor miehei, Lipozyme (Novo Nordisk, Copenhagen), catalyzes the esterification of a great variety of carboxylic acids, including short-chain, long-chain, and branched-chain acids to different types of alcohols, ranging from short-chain and long-chain alkanols to cyclic alcohols (Miller et al., 1988). In these studies almost quantitative esterification of long-chain fatty acids to long-chain alcohols has been obtained by removing the water formed under vacuum. Moreover, high rates of esterification are attained using water-immiscible solvents as reaction media rather than water-miscible solvents. A further interesting application of Lipozyme is the esterification of fatty acids to 1,2 (2,3)-isopropylidenglycerols and subsequent mild acidic hydrolysis of the isopropylidene derivative to yield monoacylglycerols as the sole reaction product (Ibrahim et al., 1988; Miller et al., 1988). Products containing high levels of monoacylglycerols have been prepared by lipase-catalyzed esterification of glycerol with rice bran oil containing large proportions of un-esterified fatty acids (Kosugi et al., 1987). Lipase-catalyzed esterification or transesterification reactions have been used for the preparation of short-chain and medium-chain esters, such as ethyl propionate, ethyl octanoate, isobutyl acetate, isoamyl acetate, phenyl 2-methylpentanoate and esters of terpene alcohols, such as menthol and geraniol (Lazar, 1985; Marlot et al., 1985; Gillies et al., 1987; Langrand et al., 1988). Such substances are used as flavoring agents. Another possible application of lipase-catalyzed esterification reactions is the synthesis of fatty acyl esters of carbohydrates, which can be
LIPASE CATALYZED REACTIONS

used as emulsifiers. Esters of sucrose, glucose, fructose, and sorbitol are reported to have been prepared in good yields by reactions catalyzed by microbial lipases, such as the lipase from *C. cylindracea* (Seino et al., 1984).

A third area is interesterification which is the process of exchanging some of the fatty acids on the triacylglyceride molecule for other fatty acids. When ester exchange is catalyzed chemically, it results in a random distribution of the entire fatty acid pool at each position on the triacylglyceride (Sonntag, 1988). When a lipase is used as the catalyst, production of a variety of desirable acylglyceride mixtures may be possible due to the specificities for either fatty acid chain length or position (Macrae, 1983b, 1989). Under moderate reaction conditions, the reaction can still proceed at rates that are acceptably rapid for industrial processing. Fats and oils with modified physical properties, particularly their melting characteristics, are commercially attractive because of potential applications.

Possible applications of lipases in the preparation of products resembling cocoa butter from inexpensive starting materials have been briefly explored. Such products, which are of interest to the confectionary, pharmaceutical, and cosmetic industries, have been prepared, for example, by interesterification of olive oil with stearic acid catalyzed by an immobilized sn-1,3-specific lipase from *Rhizopus delemar* (Yokozeki et al., 1982a). Cocoa butter substitutes have also been prepared using immobilized sn-1,3-specific lipases, e.g., by interesterification of a palm oil fraction with myristic acid using the lipase from *Aspergillus sp.* (Wisdom et al., 1984) or by interesterification of olive oil with palmitic acid using Lipozyme from *Mucor miehei* (Nielsen, 1985). Linolenic acid is an undesirable constituent of soybean oil due to its oxidative instability. Recently, the linolenic acid content of soybean oil has been substantially reduced by interesterification of this oil with lauric, palmitic or oleic acid at low temperatures (10 °C) using Lipozyme and, to a lesser extent, with pancreatic lipase (Kaimal and Saroja, 1988). Triacylglycerols enriched with ω-3 polyunsaturated fatty acids have been obtained by interesterification of cod liver oil with fatty acids or ethyl esters using Lipozyme (Haraldsson et al., 1989). Triacylglycerols, contained in common fats and oils, as well as wax esters of jojoba oil have been transesterified with various sugar alcohols in pyridine using porcine pancreatic lipase or *Chromobacterium viscosum* lipase to yield primary monoesters of sugar alcohols having excellent surfactant properties (Chopineau et al., 1988).

III. CONVENTIONAL REACTION SYSTEM FOR LIPASE CATALYSIS

As mentioned earlier, conventionally, lipases have been employed for hydrolytic reactions mainly in water-rich media. In such systems, the insoluble substrate is dispersed in the enzyme-containing aqueous solution and a lower phase ratio between substrate and water is used. Since lipase acts only at the interface, the reaction rate is a function of the interfacial area between the substrate and the aqueous phase. Accordingly, substrates must be dispersed in as fine an emulsion as possible. Simple agitation or stirring is not sufficient for substrates such as olive and milk triacylglycerols. Emulsification is done by homogenization or sonication of the
mixture of water and triacylglycerols in the presence of additives such as polyvinyl alcohol, polyethylene glycol, gum arabic, Triton X-100, and lecithin (Yamaguchi et al., 1973; Omar et al., 1987; Tahoun, 1987; Alvarez and Stella, 1989; Phillips and Pretorius, 1991). This requires mechanical energy to increase the interfacial area and the emulsifier to stabilize the resulting emulsion. Vigorous agitation of emulsified mixture is also required in the course of hydrolysis to constantly renew the surface of the oil droplets. The system also needs sodium ions to suppress enzyme inhibition by surface charge effects and calcium ions to accept a charged fatty acid, which normally inhibits lipase (Brockerhoff and Jensen, 1974; Shahani, 1975; Nishio et al., 1987b). Also, the volume fraction of substrate and water is critically important to the course of hydrolytic reaction. If a very large amount of water is used, a high degree of hydrolysis may be achieved at the expense of lower yield on a volume-time basis. On the other hand, with a small amount of aqueous phase, the reverse reaction becomes significant due to increased concentration of glycerol and decreased availability of water needed for the hydrolysis.

The aqueous emulsion reaction system is schematically shown in Figure 3. The features of this system are that it is heterogeneous and is an oil-in-water emulsion in which the water occupies a high volume fraction. The Figure shows action of soluble or immobilized enzymes in the presence of continuous mixing. In a typical hydrolysis run, oil or melted fat is dispersed in an aqueous solution containing emulsifiers and electrolytes. The dispersion is then homogenized or sonicated. A buffered enzyme solution is added and the mixture is stirred at a constant temperature, until the substrate is completely hydrolyzed or maximum degree of hydrolysis is achieved. The amount of enzyme used will depend on the reaction time desired for a degree of

![Figure 3](image-url)
hydrolysis. The enzyme is denatured even at room temperature by Cu, Fe, and Ni ions (Linfield, 1988), therefore stainless steel or glass-lined equipment must be used. Until recently, the majority of scientific reports describing lipase-mediated hydrolysis in aqueous emulsion system were confined to academic biochemical studies (Brockerhoff and Jensen, 1974; Brockman, 1984; Linfield, 1988). Also, there are several studies reported in which aqueous emulsion systems have been used to characterize the lipases from new sources (Nishio et al., 1987a; Yamamoto and Fujiwara, 1988; Jacobsen et al., 1989; van Oort et al., 1989; Alhir et al., 1990; Sugihara et al., 1990; Torossian and Bell, 1991).

Successful applications of lipases in the conventional emulsion systems have been delayed due to the following drawbacks: poor reproducibility, poor process control, lower volume-time yield, high energy requirement for preparation of substrate emulsion and continuous agitation in the course of hydrolysis, high enzyme cost because of no enzyme reuse, difficulty in product separation, incomplete hydrolysis due to substrate/product inhibition, risk of microbial contamination, and incompatibility for commercially important synthetic reactions.

IV. NOVEL REACTION SYSTEMS FOR LIPASE CATALYSIS

In the last two decades, considerable research efforts have been made at the industrial and academic levels for the commercial utilization of lipase, particularly of microbial origin, using novel reaction media and by finding newer applications for lipase catalysis. In contrast to the conventional water-rich emulsion system, lipase catalysis in a majority of the new reaction systems is carried out either in the presence of limited amount of water or practically in the absence of water. These media are either microaqueous at molecular level or microaqueous at phase level. The water-insoluble liquid substrate or the substrate dissolved in varying amounts of organic solvent, constitutes the bulk of the reaction medium. Lipase is used in free form, or in immobilized form, or is kept physically separate from substrate by microporous membranes.

The different kinds of reaction media used in various studies can be categorized based on the nature of phase heterogeneity and the type of phases as follows:

A. Macroheterogeneous systems:
   1. Liquid-liquid (immiscible solvent + water) systems
   2. Liquid-liquid-solid (immiscible solvent + water + solid) systems
   3. Solid-liquid (solid + immiscible solvent) systems
   4. Solid-liquid (solid + miscible solvent) systems

B. Microheterogeneous systems:
   1. Enzyme solubilized in mixture of water and miscible solvent
   2. Enzyme (chemically modified) solubilized in immiscible solvent
   3. Enzyme solubilized in immiscible solvent by surfactant or co-solvent

C. Specialized reaction systems:
   1. Organogels or microemulsion-based gels (MBG)
2. Various regions of surfactant/solvent/water ternary systems, such as Winsor III, lyotropic liquid crystals, and bicontinuous microemulsion systems

3. Supercritical fluids

Each of these systems has advantages and drawbacks relative to one another. Several advantages can be realized through the use of low-water content, organic media for lipase-mediated biochemical reactions. Firstly, the high solubility of lipophilic substrates in organic media could substantially reduce the volume of the reaction mixture needed to produce a given amount of product, resulting in improved volumetric productivity. Since the substrate is primarily present in the organic phase and the reaction product is transferred back into the organic phase, problems of substrate and product inhibition are reduced, if not, eliminated. Also, product recovery from the organic solution where it is relatively more concentrated is easy and less costly, compared to the product recovery from dilute aqueous solutions. Secondly, in reactions such as esterification and transesterification, where water is a product of the reactions, low-water environment shifts the equilibrium toward synthesis rather than hydrolysis. Thirdly, the insolubility or physical confinement of the enzyme in organic media permits recovery and reuse of the enzyme after filtration. Further, in several instances, it has been observed that the use of a low-water environment freezes the enzyme in a catalytically active conformation and improves its thermal stability as compared to that in aqueous media. Finally, the technological problem of bacterial contamination and undesirable water-dependent side reactions can be reduced with the use of organic media. Each of the novel reaction media listed above is discussed in this section.

A. Macroheterogeneous Systems

1. Liquid-Liquid (Immiscible Solvent + Water) Systems

This type of biphasic reaction system is composed of a water-immiscible organic phase and the aqueous phase. The aqueous phase contains the enzyme and polar reactants such as glycerol or alcohols in the case of esterification reaction. The organic phase can be either a solution of the reactant in the organic solvent or the liquid reactant alone without any solvent. Such biphasic systems not only permit high substrate and product solubility, but also drive the equilibrium-controlled reactions to completion. Since the enzyme and the product are located in different phases, product recovery is facilitated and enzyme can be recovered for reuse. The schematic of this type of reaction medium is shown in Figure 4. Such a system requires continuous mixing. Some of the factors governing the choice of solvent in such systems are high solubilizing capacity for the reactants and products, low solubility in water (octanolwater partition coefficient more than 4), minimum denaturing effect on enzyme, and low toxicity. Generally, solvents of lower polarity are less harmful to enzymes than are the more polar solvents (Carrea, 1984). Generally, iso-octane and n-hexane have been found to be most suitable solvents for enzymatic reactions.
Hydrolytic Reactions: Lipase-mediated hydrolytic reactions have been successfully conducted in this type of macroheterogeneous reaction system. Kinetics of microbial lipase-mediated hydrolysis of olive oil (Kwon and Rhee, 1987) and beef tallow (Mukataka et al., 1985) have been investigated using this type of biphasic reaction medium. The rate equation deviated from Michaelis-Menten kinetics when the bulk concentration of oil was considered; however, the rate data obeyed the kinetic equation when the interfacial area between the two-phases was taken into consideration. The stability of lipase in this medium is dependent on the substrate concentration (Kwon and Rhee, 1986). Also, the hydrolysis rate in this medium increases in proportion with the olive oil content up to 90% v/v, as compared to 5% in a conventional aqueous emulsion system (Kwon et al., 1987a). The enzyme stability increased in proportion with concentration of oil in organic solvent. Mukataka et al. (1987) studied hydrolysis of palm oil and beef tallow by *C. cylindracea* lipase for practical applications in this medium using a high substrate concentration. For both palm oil and beef tallow, a percentage of hydrolysis higher than 98% was achieved in the 20% *iso*-octane system at a substrate concentration of 50%. However, when substrate concentration was higher than 50%, the final value of hydrolysis decreased. Approximately 60% of lipase activity was recovered by ultrafiltration. Khor et al. (1986) established optimum conditions for lipase-catalyzed hydrolysis of palm oil in this system using a single-variable approach. Lipase from *C. rugosa* (*C. cylindracea*) exhibited optimum activity at 37 °C and at pH 7.5. The optimal oil-to-hexane ratio was 2:1. The use of biphasic system is particularly helpful for the hydrolysis of animal fats (solid fat). Because of their high melting points, hydrolysis of animal fat in aqueous systems is more difficult than that of liquid oils. In the presence of 5–10% *iso*-octane, beef tallow and pork lard at 50% concentration were hydrolyzed below their melting points up to 94–97% in 24 hr using *C. rugosa* lipase (Virto et al., 1991).

Linfield and co-workers (1984a,b, 1988) investigated the hydrolysis of fats and oils using approximately equal proportions of enzyme solution as an aqueous phase and
liquid substrate as an organic phase. A 95–98% hydrolysis of tallow, coconut oil, and olive oil at 26–40 °C was achieved in 72 hr using *C. rugosa* lipase. The kinetics of lipolysis were found to be approximately of first order in the substrate concentration. From the hydrolysis data, it was empirically found that the percentage of free fatty acids formed was a linear function of the logarithm of reaction time and logarithm of enzyme concentration. They also observed that addition of hydrocarbon solvents and nonionic surfactants in this system led to an adverse effect in the hydrolytic reaction. Omar *et al.* (1987) studied hydrolysis of tallow by *Humicola lanuginosa* lipase in a similar biphasic system of liquified fat and aqueous enzyme solution. They observed that beef tallow was hydrolyzed up to 65% in 72 hr at 45 °C in a system containing about equal proportion of fat and buffer. Addition of *n*-heptane in this system increased the degree of hydrolysis up to 95%.

A technologically feasible biphasic system for the recycling of lipases in continuous fat hydrolysis has been developed (Buhler and Wandrey, 1987, 1988). It exploits the fact that lipases accumulate at the phase boundary. The process employs two stirred-tank reactors and two continuously operating centrifuges. The operating conditions were selected such that about 90% of the pure aqueous phase containing glycerol and about 90% of the pure fat phase containing fatty acids were separated. It was possible to recycle about 90% of the enzyme together with the interfacial layer. The feasibility of such a process was demonstrated with a continuous hydrolysis of soybean oil up to 98% and a volume-time yield of 11.4 kg fatty acid per liter per 24 hr.

**Transesterification Reactions:** There have been few studies in which reversal of hydrolytic reactions, i.e., esterification and transesterification have been addressed. Kwon and Rhee (1985) have evaluated different organic solvents for their effects on the stability and activity of *R. arrhizus* lipase for interesterification of fats and oil in biphasic systems. Di-*iso*-propyl ether and *iso*-octane were found to be superior for the interesterification reaction. Glycerolysis reactions have been studied in some detail for the production of monoacylglycerols from beef tallow and palm oil using this type of reaction medium (McNeil *et al.*, 1990; McNeil and Yamane, 1991; McNeil *et al.*, 1991). The systems were composed of 18.3% glycerol, 0.7% water, and 81% fat or oil (molar ratio of glycerol to fat was 2). Of the several enzyme tried, lipase from *Pseudomonas fluorescens* was the most effective, giving the highest yield of monoacylglycerols. Also, combinations of lipases from different sources were more effective than a specific lipase alone. The yield of monoacylglycerols was greatly influenced by the reaction temperature. At higher temperatures (48–50 °C), a yield of approximately 30% monoacylglycerols was obtained; while at lower temperatures (38–46 °C), a yield of about 70% monoacylglycerols was obtained (McNeil *et al.*, 1990). The authors designated the temperature below which a high yield of monoacylglycerols could be obtained as the critical temperature (*T*$_c$). In a further investigation (McNeil *et al.*, 1991), it was observed that the value of *T*$_c$ depended on the fat type and varied between 30 °C and 46 °C for naturally occurring oils. Based on this observation, McNeil *et al.* (1991) developed a lipase-mediated glycerolysis process employing two-step temperature programming during
reaction. With an initial temperature of 42 °C for 8–16 hr followed by incubation at 5 °C for up to 4 days gave a yield of about 90% monoacylglycerols from beef tallow, palm oil, and palm stearin.

Despite some advantages, the use of lipase in a two-phase system cannot be extended to catalyze the esterification, since the location of enzyme is not different from that in an aqueous emulsion. A real obstacle to the use of lipase even for hydrolysis in a two-phase system is the small interfacial area. This causes the reaction to be mass transfer-limited. The observation that water droplets formed by agitation are much larger than oil droplets formed by emulsification indicates that increase of interfacial area is still desirable (Han et al., 1988). However, vigorous agitation to reduce the droplet size may lead to deactivation of lipase due to shear force. This may also increase the energy cost. Therefore, methods are needed that could retain the advantages of a two-phase reaction system while alleviating its drawbacks.

2. Liquid-Liquid-Solid (Immiscible Solvent + Water + Solid) Systems

This type of macroheterogeneous medium is characterized by existence of multiple phases including an aqueous liquid phase, an organic liquid phase, and a solid phase. The aqueous phase may be either a solution of polar reactant in water or the polar liquid reactant by itself. The organic phase may be either a solution of non-polar reactant in a solvent or the reactant in the absence of any solvent. The solid phase may be either a lipase immobilized on solid support or a microporous membrane containing the enzyme. Compared to the biphasic systems, two additional benefits may be gained with this reaction medium. Firstly, as the biphasic systems cause denaturation of the enzyme because of adsorption at liquid-liquid interface, immobilization of the biocatalyst on a solid support may alleviate this problem. Secondly, the reuse of the enzyme through recycling is greatly facilitated. The schematic of this type of reaction medium is shown in Figure 5.

Hydrolytic Reactions: This type of reaction medium is suitable, particularly, for hydrolytic reaction of lipase because of the presence of significant amount of water in the systems. Also, inhibition against the enzyme is less as the product is transferred into the organic phase. Most studies of the hydrolysis of triacylglycerols in this type of medium have used membrane-type bioreactors with few exceptions. Kwon et al. (1987b) studied hydrolysis of triacylglycerol in iso-octane-water system using lipases from *C. rugosa* and *R. arrhizus* immobilized by entrapment with photo-cross linkable resin prepolymer. The ratio of solvent to water phase was 8:2 with 10% v/v olive oil in iso-octane. Lipase entrapped with hydrophobic gel (ENTP-3000) exhibited the highest activity. As the degree of hydrophobicity of the immobilization matrix increased, the Michaelis-Menten reaction velocity $V_{\text{max}}$ of the lipase entrapped was increased. In comparison to free enzyme, the initial hydrolysis rate with the immobilized lipase was lower. However, both free and immobilized lipases showed almost the same degree of hydrolysis after 25 hr of reaction. Similarly, hydrolysis of triolein has been studied using immobilized *C. cylindracea* lipase on hydrophobic
and hydrophilic zeolites in a system containing 1.5 g of enzyme preparation in a mixture of 21% (v/v) of buffer (Lie and Molin, 1991). Triolein was hydrolyzed up to 70% with the lipase immobilized on hydrophobic zeolite; whereas the hydrolysis was completely blocked by hydrophilic zeolite.

Membrane bioreactors offer promising possibilities for an efficient integration of bioconversion and separation processes. The possibility of performing biocatalytic conversions between two immiscible phases separated by a membrane makes this technology, particularly, interesting for the oils and fats industry. Recently, several approaches involving a membrane bioreactor (MBR) have been tried. Membrane bioreactors can be operated in different ways. For the hydrolysis of fats and oils, mainly microporous (0.1–0.4 μm) hydrophobic membranes have been used (Hoq et al., 1985a, b, 1986; Taylor et al., 1986). The lipase is adsorbed onto polypropylene membranes and the interfacial enzyme concentration is dependent on the purity of the enzyme separation used (Hoq et al., 1985b). Using hydrophobic membranes, the enzyme must be immobilized on the membrane side that will be exposed to the aqueous phase. The membrane will be saturated with oil, which results in a very poor diffusion of the substrate water and of the product glycerol through the membrane and, consequently, in low reaction rates if the enzyme is immobilized on the oil-phase side of the
membrane. By properly balancing the pressure on either side of the membrane, it is possible to keep the two phases completely separated. Separation of the resulting glycerol solution and free fatty acids is realized in the membrane unit. Using a thermostable lipase immobilized on a hydrophobic membrane, Taylor et al. (1986) succeeded in passing tallow through the membrane. The two phases were separated by settling the mixture after which the aqueous phase could be recycled.

Alternatively, the enzyme can be immobilized (via ultrafiltration) onto a hydrophilic membrane on the oil side of the reactor (Pronk et al., 1988). For such a configuration, the following mechanism is required for the lipolysis reaction. First, water must diffuse through the membrane to the enzyme active site. Lipolysis then takes place and the resulting glycerol molecule must diffuse back through the membrane to the aqueous effluent stream. Failure of these two diffusion processes to take place would result in a rapid build-up of the glycerol concentration in the vicinity of the enzyme and, consequently, decrease the reaction rate. Pronk et al. (1988) demonstrated a quantitative diffusion of glycerol through the membrane during the total recycle lipolysis of olive oil. Whether the reaction rate is transport-controlled or kinetically controlled is not yet known, but in either case it would be dependent on specific enzyme activity, membrane thickness and morphology.

In most cases, the stability of the immobilized lipases was high. Half-lives in the order of several months have been reported. It is generally accepted that lipolysis can be described by Michaelis-Menten kinetics if the substrate concentration is replaced by the interfacial surface area (Macrae, 1983a). In an MBR, this area is determined by the total membrane area. Kloosterman et al. (1988) added enzyme dissolved in a small amount of water to the oil phase, so that the co-substrate water and interfacial area (emulsion) become available to the enzyme. This approach resulted in high rates of hydrolysis. In the membrane unit, glycerol moves to the aqueous phase and water to the oil phase. Using this approach, only 5–10% (v/v) water had to be added to the oil phase to obtain a maximum rate of hydrolysis at 40°C. Whether this route offers better opportunities for commercialization in relation to the immobilized enzyme system will depend on the stability/reusability of the enzyme and on the reaction rate of both systems.

**Esterification Reactions**: During esterification, water is produced while glycerol is consumed. If esterification reactions are conducted in emulsion systems, then water activity is increased during the reactions. In contrast, using an MBR, there are several approaches to control water activity. Hoq et al. (1984) advocated the use of a microporous hydrophobic membrane onto which lipases could be adsorbed. The adsorbed lipases desorbed relatively easily in glycerol solutions (97%). Therefore, lipase was added to the glycerol solution, which was pumped across the membrane surface. In this way, an equilibrium was established between surface-adsorbed enzyme and enzyme in free solution. On the opposite side, free fatty acid (oleic acid) was circulated through the reactor. The water activity in this system was controlled by circulating the glycerol solution through a continuous dehydrator.

Hydrophilic membranes also can be used in cases where the enzyme is physically immobilized via ultrafiltration on the fatty acid/acylglycerol side of the membrane.
A glycerol solution with a given water content is pumped along the opposite side of the membrane. The water activity of this phase can be controlled either by a molecular sieve or by evaporation (Kloosterman et al., 1988). As for lipolysis in the previous examples, the reaction is limited to the oil-water interface at the membrane surface. Direct addition of the lipase dissolved in a small amount of water to the oil phase also may be considered (Kloosterman et al., 1988). This water phase will equilibrate with the glycerol solution on the opposite side of the membrane. For this to occur, the glycerol and water activities in the droplets containing enzymes must equilibrate with those in the glycerol feed solution. Thus, by this approach an emulsion reaction is combined with a membrane process to control the water activity in the vicinity of the enzymes.

### 3. Solid-Liquid (Solid + Immiscible Solvent) Systems

This type of reaction medium is composed of a large proportion of the organic solvent phase containing the non-polar reactant, and a solid phase comprised of either dry enzyme powder or enzyme immobilized on the microporous support. A non-polar liquid substrate may be used as the organic phase without addition of any organic solvent, as well. The system contains water only at the molecular level. A small amount of water required for the catalytic activity of lipase is associated with dry enzyme powder or microporous support, and with the organic solvent. Depending upon the type of reaction, the water is either continuously recharged or removed from the system. This type of system is primarily used for the synthetic and transesterification activity of lipase with a few studies on hydrolytic reactions. The extremely low water content in this system facilitates shifting of equilibrium toward synthetic activity of lipases, which is difficult to realize in water-rich systems. The schematic of this type of biphasic macroheterogeneous reaction medium is shown in Figure 6.

**Hydrolytic Reactions:** As mentioned earlier, the system is more suitable for esterification or transesterification reactions. However, hydrolytic reaction of lipases has been investigated in a few studies. Continuous hydrolysis of olive triacylglycerols in di-isopropyl ether using *R. arrhizus* mycelia as a source of lipase has been studied (Bell et al., 1981). Typically a packed-bed reactor containing 1 g of mycelia fed at 1 mL/min with a solution of 2.5% (w/v) substrate in the solvent gave a fatty acid yield of 45% at 30 °C. The optimum water concentration was found to be 0.17% (w/v). The loss of enzyme activity was 0.6–1.0% /hr at 30 °C. Kang and Rhee (1988,1989a, b) have conducted a detailed study on the hydrolysis of olive oil by an immobilized lipase from *C. rugosa* in an organic solvent. The lipase was immobilized by adsorption on various supports which could contain water available for the hydrolysis of olive oil in organic solvent. The lipase immobilized on swollen Sephadex LH-20 could almost completely hydrolyze 60% (v/v) olive oil in iso-Octane. The hydrolytic reaction did not follow Michaelis-Menten kinetics. Maximum activity was obtained at pH 7. The optimum temperature shifted towards higher values with an increase of olive oil concentration. Bilyk et al. (1991) studied the
LIPASE CATALYZED REACTIONS

hydrolysis of different triacylglycerols in hexane using Rhizomucor miehei lipase, immobilized on anion exchange resin in the presence of amines. The presence of secondary amines greatly increased the extent of hydrolysis. Lipase, in the absence of amine, hydrolyzed the tallow up to 76% in water-saturated hexane. The addition of secondary amines to tallow solution in hexane increased the hydrolysis to 95% under similar conditions. Recently, a moistened caryopses of oat (Avena sativa) has been used as a natural lipase bioreactor for hydrolysis of oil (Lee and Hammond, 1990). The moistened (about 20% of its weight) caryopses of oat, when immersed in oil or in hexane containing oil, hydrolyzed the oil without any positional specificity. The optimum temperature was about 40 °C.

Esterification and Transesterification Reactions: After the first report (Zaks and Klibanov, 1984) that described the catalytic activity of dry lipase in organic media at very high temperature, several studies were conducted using suspension of solid powdered enzyme in organic solvent for synthetic reaction of lipases. Boyer et al. (1990) studied the esterification activity of solid Mucor miehei lipase using oleic acid and decanol in heptane. The activation of the enzyme was affected by its swelling with water. By controlling the water content a conversion of up to 94% was obtained in 1.5 hr. Lipase-mediated alcoholysis of sunflower oil in anhydrous petroleum ether was examined using lipase powder from P. fluorescens (Mittelbach, 1990). Significant proportions of mono- and diacylglycerols were produced with methanol. Chopineau et al. (1988) studied transesterification reactions between a number of sugar alcohols and various plant and animal fats in pyridine using solid lipase from Chromobacterium viscosum. The reaction produced highly surface-active primary monoesters of sugar alcohols and fatty acids. Elliott and Parkin (1991) studied

FIGURE 6  Schematic of solid-liquid (solid + immiscible solvent) macroheterogeneous reaction medium. The liquid phase is a solution of the substrate in an organic solvent or the pure liquid substrate. The solid phase consists of dry enzyme powder or enzyme immobilized on a solid matrix. Only molecularly dissolved water is present.
acyl-exchange reactions between butteroil and fatty acids in anhydrous media using solid pancreatic lipase. Optimum temperature and pH for the reaction were found to be 70 °C and 6.5, respectively.

Several immobilized lipases have been used to catalyze transesterification reactions in organic solvents for the modification of fats and oils (Lilly and Dunnill, 1987; Sawamura, 1988; Schuch and Mukherjee, 1988; Tanaka et al., 1988; Bloomer et al., 1990; Chang et al., 1990). Macrae (1983b) reported that *Rhizopus niveus* lipase adsorbed on kieselguhr had been used for interesterification for 400 hr with almost no loss of activity. The substrates, palm oil midfraction and myristic acid, were dissolved in petroleum ether (b.p. 100–120 °C) saturated with water. In a similar example *Mucor miehei* lipase on Hyflo SuperCel was used to catalyze the interesterification of palm oil midfraction and stearic acid in packed-bed reactor at 50 °C (Macrae, 1985). The enzymatic activity decayed exponentially with a half-life of about 60 days. Recently, lipase-mediated reactions in this type of reaction medium has comprehensively been reviewed (Malcata et al., 1990).

**Solid-Liquid (Solid + Miscible Solvent) Systems**

This type of macroheterogeneous biphasic system is characterized by a solid phase comprised of enzyme adsorbed on a solid support or entrapped in a microporous carrier, and a homogeneous liquid phase comprised of water and miscible organic solvent. The mixed liquid phase increases the solubility of polar or non-polar reactants in the medium. Solvents used include ethanol, acetone, dimethyl sulfoxide, acetonitrile, and tetrahydrofuran. The major criterion for the selection of the organic solvent is the ability to maintain the catalytically active conformation of the enzyme without stripping the essential hydration shell. With small additions of these solvents, enzymes usually retain their activity, but at higher solvent concentrations inactivation of enzyme is observed. The schematic of this type of macroheterogeneous biphasic systems is shown in Figure 7.

The system may be suitable for lipase-mediated synthetic reactions because of the ability of this type of medium for solubilizing both polar and non-polar reactants. However, there are no examples in the literature where lipase-mediated reactions

![FIGURE 7 Schematic of solid-liquid (solid + miscible solvent) macroheterogeneous system. The enzyme is immobilized in a solid matrix or adsorbed on a solid support. The liquid phase is a mixture of water and a polar organic solvent and contains the substrate.](image-url)
LIPASE CATALYZED REACTIONS

have been carried out in such a system. However, a few other enzymes have been studied in this type of system. A column containing immobilized glucose oxidase was operated continuously for 14 days in a 20% (v/v) acetone solution without any considerable decline in enzyme activity (Alberti and Klibanov, 1982). In another example, Yokozeki et al. (1982b) carried out a transglycosylation reaction using immobilized whole cells (Enterobacter aerogenes). This biocatalyst retained its original activity after 35 days of operation in 40% (v/v) dimethysulfoxide.

B. Microheterogeneous Systems

1. Enzyme Solubilized in Mixture of Water and Miscible Solvent

This reaction medium is composed of a single liquid phase with different proportions of water and a miscible organic solvent, with the substrate and the enzyme dissolved in the mixed solvent. Enzymatic reaction in such media offer advantages similar to those described in the preceding section. The reaction in this medium is not limited by mass transfer consideration and reactants of different polarity can be conveniently dissolved. However, there is some degree of difficulty in product separation. Further, an organic solvent at concentrations greater than 50–70% may strip the hydration shell of the enzyme causing it to denature (Arakawa and Goddette, 1985; Guagliardi et al., 1989). However, modest concentrations of some organic solvents enhance the stability of enzymes (Butler, 1979; Freeman, 1984). The schematic of the reaction system is shown in Figure 8.

Hydrolytic Reactions: A reaction system containing lipase from Pseudomonas sp. in water/acetone solvent mixture was used for resolution of rac-α-acyloxystannanes to prepare optically active α-hydroxystannanes (Itoh and Ohta, 1990; Itoh et al., 1990). A similar solvent system was used for hydrolysis of prochiral diacetates to optically active paraconic acid (intermediate for streptomycin) by pig pancreatic lipase (Mori and Chiba, 1989).

![Figure 8](https://example.com/figure8.png)

FIGURE 8 Schematic of systems with enzyme dissolved in mixture of water and miscible solvent. The solvent mixture can dissolve both the substrate and the enzyme.
Transesterification Reactions: Lipases have been used in water-miscible solvent systems for transesterification reactions. *Pseudomonas fluorescens* lipase dissolved in the mixed solvent system of water, tetrahydrofuran and pyrene has been used for regioselective esterification of sugars such as methyl rhamnopyranoside (Ciufreda *et al.*, 1990). Lipase from *Chromobacterium viscosum* has been studied using water/acetone solvent system for the regioselective esterification of chloramphenicol at primary hydroxy group (Ottolina *et al.*, 1990).

Water-miscible organic solvent is not used for lipase-mediated reactions with triacylglycerols. This may be due to the less than desirable stability of the enzyme in these systems and comparatively lower solubility of the highly lipophilic substrates.

2. Enzyme (Chemically Modified) Solubilized in Immiscible Solvent

Chemical modification of enzymes has potential biotechnological utility. The structural stability, performance in the hydrophobic environment, and ease of recovery and reuse of enzyme may be improved by such chemical modifications as reductive alkylation of lipase (Kaimal and Saroja, 1989), polyethylene glycol- modified lipases (Inada *et al.*, 1990), and lipid-coated lipase (Okahata and Ijiro, 1988). Generally, this type of microheterogeneous reaction system is comprised of water-immiscible organic solvent, in which enzymes are made soluble and stable by chemical modifications. The system contains water only in the molecularly dissolved state. The essential water is associated with the modified enzyme preparation. The advantage of this reaction system is the enhanced solubility of highly lipophilic substrates and the enzyme in the medium. The reaction is no longer limited by interfacial area or diffusion. The schematic of the reaction system is shown in Figure 9.

A new approach in biotechnological processes is to use lipase modified with polyethylene glycol (PEG) which has both hydrophilic and hydrophobic properties. The PEG-lipase is soluble in organic solvents such as benzene and chlorinated hydrocarbons and exhibits high enzyme activity and stability in organic solvents. The PEG-lipase catalyzes the reverse reaction of hydrolysis in organic solvents: ester synthesis and ester exchange reaction (Inada *et al.*, 1986, 1990; Baillargeon and Sonnet, 1988; Takahashi *et al.*, 1988). The PEG-lipase can also be conjugated to

![FIGURE 9 Schematic of microheterogeneous systems with chemically modified enzyme solubilized in water-immiscible solvent. The substrate is dissolved in the solvent medium and is directly accessible to the enzyme.](image)
magnetite ($\text{Fe}_3\text{O}_4$). The magnetic lipase catalyzes ester synthesis in organic solvents and can be readily recovered by using magnetic field without loss of enzymic activity (Tamaura et al., 1986; Takahashi et al., 1988; Inada et al., 1990). The PEG-lipase catalyzed the ester-exchange reactions between an ester and an alcohol, an ester and an acid and between two esters (Takahashi et al., 1985). These reactions occur not only in organic solvents but also in hydrophobic substrates in the absence of any solvent. This suggests that the modified lipase is useful for many practical applications. One of them is the modification and formation of fats and oils. PEG-lipase catalyzed the interesterification reaction between trilaurin and triolein in the absence of solvents at 58°C (Matsushima et al., 1986). Dilauroyl-monoleoyl glycerol and monolauroyl-dioleoyl glycerol were formed as the products of the reaction. As a result, the melting temperature of the mixture of two substrates decreased from 33–36°C to 11–13°C. Similarly, interesterification between hard fats and olive oil by PEG-lipase resulted in modified triacylglycerols with substantially lower (11–14°C less) melting point (Matsushima et al., 1986; Inada et al., 1990).

Kaimal and Saroja (1989) used reductive alkylation of porcine pancreatic lipase with butyraldehyde and acetone to enhance activity of the lipase in organic solvent. They found about 50% increase in the maximum reaction rate. The esterification activity of \textit{C. cylindracea} lipase in organic solvent was markedly enhanced by the reduction of disulfide bonds with dithiothreitol without appreciable loss of hydrolytic activity (Kawase and Tanaka, 1988, 1989). A new type of organic solvent-soluble lipase preparation was developed using lipid-coating of the enzyme (Okahata and Ijiro, 1988). The lipid-coated lipase was prepared by mixing aqueous solutions of enzyme and synthetic dialkyl amphiphiles; it was insoluble in water but soluble in most organic solvents and showed a high activity for the synthesis of di- and triacylglycerols from monoacylglycerols and fatty acids in non-aqueous and homogeneous organic solvent systems. In another study, the lipid-coated lipase vigorously catalyzed the reaction of enantioselective esterification of racemic alcohols in this type of reaction system (Okahata et al., 1988).

3. Enzyme Solubilized in Immiscible Solvent by Surfactant or Co-Solvent

These microheterogeneous systems are characterized by having a continuous organic liquid phase containing non-polar reactants, and a dispersed microaqueous liquid phase containing polar reactants and enzyme solution. As in the case of biphasic macroheterogeneous systems, the enzyme in this system is also spatially separated from the organic solvent, but it is free from a diffusioned limitation. The reason is that the separation between aqueous and organic phase occurs at the 'molecular level' rather than at the 'phase level'. Unlike the macroheterogeneous systems these systems are also optically transparent and thermodynamically stable mixtures of water and immiscible solvent. In this type of microheterogeneous system, enzyme is solubilized in an immiscible organic solvent either by micellar solution of surfactants (named as water-in-oil microemulsion or reverse micellar medium) or by hydrogen-bonded aggregates of water and polar co-solvent (named as detergentless microemulsion or ternary system of hydrocarbon/polar-solvent/water).
Figure 10 shows the schematic of this type of microheterogeneous reaction system. Reverse micellar system is a novel approach to biphasic aqueous-organic biocatalysis. In such a system, enzyme is solubilized in the microaqueous phase inside the surfactant micelles. The reverse micelles protect the enzyme from solvent-induced denaturation and provide the enzyme with the water, essential for its structural stability. Water-insoluble substrates and products are solubilized in the bulk solvent, providing a large interface for the enzymatic reaction.

Reverse micellar systems have been extensively studied in the last two decades for various reasons ranging from fundamental understanding of in vivo enzyme action to application in the area of protein separation and enzymatic reactions. As mentioned earlier, the scope of this paper is limited to applications of reverse micellar systems for lipase catalysis. Different aspects of enzyme catalysis in reverse micellar media, namely, biotechnological applications (Leser et al., 1987; Martinek et al., 1987a, b), fundamentals of micellar enzymology (Levashov et al., 1984; Martinek et al., 1986; Luisi and Steinmann-Hoffman, 1987), and general aspects such as structure of reverse micellar media, preparation of the system, and experimental procedures (Luisi and Wolf, 1982; Luisi, 1985; Luisi and Laane, 1986; Luisi and Magid, 1986; Laane et al., 1987) have comprehensively been reviewed. Lipase-mediated reactions in reverse micellar media have a great potential for food applications. Use of reverse micellar media greatly facilitates the lipolytic reactions. The substrates which have to be emulsified prior to enzymatic hydrolysis can be solubilized to a large extent in the oil-continuous phase while the enzyme is hosted in the reversed micellar phase. The enormous interfacial area that is possible in reverse micelles tremendously
improves enzymatic activity. Since the substrate is mainly concentrated in the oil-continuous phase, substrate inhibition problems are mitigated. The first report (Misiorowski and Wells, 1974) on the catalytic activity of lipolytic enzyme in reverse micellar media initiated the subsequent research in the field of reverse micellar enzymology. Since then several enzymes including various microbial lipases have been studied in reverse micelles.

Hydrolytic Reactions: A reverse micellar system composed of Aerosol-OT (bis (2-ethylhexyl) sodium sulfosuccinate) as a surfactant and iso-octane as a non-polar solvent is well characterized and widely used system for many enzyme-catalyzed biochemical reactions. This system has been used in a number of studies of lipase-mediated hydrolysis of triacylglycerols (fats and oils) to glycerol and fatty acids (Han and Rhee, 1985, 1986; Kim and Chung, 1989) and partial acylglycerols (Holmberg and Osterberg, 1988). Batchwise hydrolysis of olive oil by lipase from C. rugosa revealed that the substrate (5% v/v) could be almost completely hydrolyzed at R value of 10 (R value is the molar ratio of water to surfactant in the reverse micelle) and at Aerosol-OT concentration of 100 mM. At the end of the reaction, fatty acids produced could be recovered with high yield by adding water and acetonitrile, centrifuging the mixture and collecting the upper phase (Han and Rhee, 1985). In the hydrolytic reaction of olive oil, lipase activity and stability in reverse micelles were highly dependent on the R value. The R values which maximized the initial velocity and stability were 10.5 and 5.5, respectively (Han and Rhee, 1986). Lipase in this system was not inhibited even at a substrate concentration of 40% (v/v). This was in contrast to the behavior in aqueous macroemulsions where substrate inhibition could be observed at 3–5% (w/v) of substrate concentration. The temperature-activity and pH-activity profiles of the enzyme were similar to that in buffer solution. Among the eight organic solvents tested, iso-octane was most effective for the hydrolysis of olive oil in reverse micelles. Similar results were obtained by Kim and Chung (1989) who investigated R. arrhizus lipase-mediated hydrolysis of palm kernel olein in Aerosol-OT/iso-octane reverse micelles. The lipase was the most effective at R = 13 when hydrolysis was carried out at pH 7 and 30°C. Among the various additives tested, glycine, histidine, and glycerol increased the initial rate of reaction. In the presence of histidine and casein, the fatty acid production was increased about 1.5-fold after 48 hr reaction.

Lipase reaction in reverse micellar system is expected to be affected by the water concentration because its content in the system is usually below 2% (v/v) of the total reaction mixture. In fact, the initial water concentration affected significantly the equilibrium of hydrolytic reaction of lipase. The equilibrium fractional conversion of ester bond to fatty acid and alcohol moiety increased in proportion with the initial water content in reverse micelles (Han et al., 1987). While hydrolytic reactions in water are usually considered to be a pseudo-first-order reaction, the low-water environment in reverse micelles necessitated the reaction to be modelled as a two-substrate reaction. Although effect of water on equilibrium and rate parameter may be inferred from the two-substrate kinetics, other factors such as the amount of protein in water pool, ionic strength, pH, ionic species, and temperature have
greater influence on the reaction by altering the properties of water. Han et al. (1990) studied the activity of C. rugosa in reverse micelles at various concentrations of water and the enzyme. For low water content (below \( R = 6 \)), the activity increases with increasing water content indicating the requirement of a minimum amount of water for the full expression of enzymatic activity. The minimal \( R \) value for obtaining maximal activity depends on the enzyme concentration: the higher the enzyme concentration, the higher the optimum value of \( R \).

Holmberg and Osterberg (1988) studied hydrolysis of palm oil using sn-1(3)-specific lipase (R. delemar) for preparation of monoacylglycerols in Aerosol-OT/iso-Octane reverse micelles. Monoacylglycerols in 80% yield was obtained in 3 hr at 35°C. The molar ratio \( R \) was critical in determining the amount of fatty acids and monoacylglycerols obtained as a results of the reaction. The optimum \( R \) was found to be 12 for monoacylglycerol yield at pH 7.0 and 35°C.

Type of surfactant used in reverse micellar media could greatly influence the lipase activity. Enzymatic hydrolysis of palm oil by Rhizopus sp. lipase has been studied using different composition of reverse micelles based on non-ionic surfactant, pentaeethylene glycol monododecyl ether in iso-octane (Stark et al., 1990). The rate of reaction decreased as the water content of the reaction medium was increased. The non-ionic surfactant was found to be unsuitable for enzymatic reactions since only partial hydrolysis was obtained in all experiments. In another study (Kery et al., 1989), six ethylene oxide-based non-ionic surfactants were tested for their suitability to mediate lipase action on olive oil in reverse micellar media. Only with Slovasol El (ricine oil + 20 molecule of ethylene oxide), it was possible by the lipase to catalyze hydrolysis of the substrate over a wide range of iso-octane and water content. The possible advantage of this type of system over Aerosol-OT based system is that the stable systems for catalysis can be prepared over a wide range of concentration of aqueous and non-aqueous phases.

Efforts have been made to replace biocompatible surfactant and/or solvent in the typical Aerosol-OT/iso-octane reverse micellar media to develop more practical reaction systems. Lipase-mediated hydrolysis of triacylglycerols in reverse micelles formed by soybean lecithin in iso-octane has been studied (Schmidli and Luisi, 1990). The reaction rate was found to be diffusion-controlled. The maximum ratio was found to be at low \( R \) value of 2.2. The temperature stability of the lipase in lecithin reverse micelles was higher than aqueous solution. The initial rate was highest at 60 °C. A solvent-free reverse micellar medium of purified lecithin or crude soy lecithin preparation as the surfactant and liquid triacylglycerol as the substrate and continuous non-polar phase for lipase-mediated hydrolytic reaction have been developed in our laboratory. In a concurrent study, Chen and Pai (1991) used similar lecithin reverse micellar media for hydrolysis of milk triacylglycerols using C. cylindracea lipase. The initial rate was maximum at 55 °C. The maximum activity was observed between pH 4 and 6. The molar ratio (\( R \)) of 10 produced maximum reaction rates. O'Connor et al. (1991) studied surfactant-free microemulsion media of n-hexane/iso-propanol/water for C. cylindracea lipase-mediated hydrolysis of methyl palmitate. Since this system does not require the presence of surfactant for its formation, it offers the advantages of simple product separation and enzyme reuse. However, only 9% of the substrate methyl palmitate ester could be hydrolyzed in
LIPASE CATALYZED REACTIONS

This system. Thus, poor intrinsic catalytic activity and stability of enzymes may make the surfactant-free system less than desirable.

Many of these studies have focused on the initial rate based on 20–60 min reaction time for assessing the influence of reaction parameters on hydrolytic reaction. During the hydrolysis of triacylglycerols, water is consumed and proton-donating species (fatty acids) are generated. As a result, the microenvironment in the aqueous pool is continuously changing from the initial conditions. So, for the practical utility of such systems, the relationship of reaction parameters with initial rate as well as with the extended time-course of the hydrolysis should be examined. Also, studies reported in the literature have been conducted using a single-factor approach which does not provide important information regarding interactive effects of reaction parameters on hydrolysis. Nevertheless, this reaction system seems to be promising for the enzymatic hydrolysis of fats and oils since it does not require energy to promote and maintain interfacial area for catalysis. Also, the system is chemically well defined. Although it is certain that reverse micelles may open a new way to the industrial application of lipase, a principal hurdle to the successful application may be the difficulty in recovering the enzyme, if necessary, from the reaction mixture at the end of the reaction. Appropriate enzyme reactors which are compatible with the organic solvent used, and thus permit the continuous operation, may contribute to economically feasible applications of reverse micelles.

The emulsion-based conventional enzymatic hydrolysis of fats and oils (triacylglycerols) has not been successful as an alternative process to physicochemical hydrolysis of fats. A reverse micellar system of Aerosol-OT/iso-octane was studied for hydrolysis of various triacylglycerols (TAG) using *Rhizopus javanicus* and *Candida cylindracea* lipases (Patel et al., 1995). The influence of various reaction parameters on the hydrolytic reaction was investigated using multi-variable experiments (Patel et al., 1996a). A solvent-free micellar medium, suited to food application, was developed and investigated using model substrates and milk fat (Patel et al., 1996b). With the Aerosol-OT reverse micellar system, up to 98% of substrate could be hydrolyzed by using non-specific lipase. The hydrolysis reaction obeyed Michaelis-Menten kinetics. Kinetic constants were related to enzyme source, reaction conditions, and physicochemical characteristics of substrates. The initial rate (IR) and the degree of hydrolysis (DH) of TAG were most significantly affected by pH, temperature, and molar ratio of water to surfactant (R). The IR was most influenced by the interactive effect of pH with temperature, R, and Aerosol-OT concentration. The DH was most influenced by the interactive effects of reaction temperature with R and pH. Optimum reaction conditions for hydrolysis in this system were 22 °C, 140 mM Aerosol-OT, pH 6.8, and R = 14. Hydrolysis of TAG in solvent-free lecithin reverse micelles was significantly influenced by temperature and R. Both lipases exhibited unusually high thermal stabilities in this system as evidenced by the significant activity at 74 °C and 64 °C, respectively. Unlike the Aerosol-OT system, the optimum reaction conditions in this system were highly dependent on the physicochemical properties of substrates (Patel et al., 1996c). Degree of hydrolysis was increased 2-fold by maintaining R value or was increased 2.6-fold by gradually increasing the R value of the system during hydrolysis. Positional selectivity of the two lipases varied with type of reverse micelles. The content and the composition of various components in milk fat
hydrolysates prepared in the solvent-free lecithin reverse micellar system were significantly influenced by reaction temperature and R. The optimum reaction conditions necessary for favoring the production of short-chain fatty acids, mono- and diacylglycerols, and specific regio-isomers of partial acylglycerols have been defined. Thus, it may be possible to use a solvent-free system in the modification of milk fat to impart desirable multiple functionality by selecting appropriate reaction conditions.

Reverse micellar system of Aerosol-OT in iso-octane can be used for lipase-catalyzed hydrolysis of triacylglycerols (TAG). In this system, TAG at a concentration of 20% (v/v) were hydrolyzed up to 50–60% by Rhizopus javanicus lipase and up to 95–98% by Candida cylindracea lipase. The hydrolysis reaction obeyed Michaelis-Menten kinetics. As evidenced by the linear relationship of initial rate with enzyme concentration, the reaction rate for TAG hydrolysis in reverse micelles was kinetically controlled and not limited by mass transfer consideration. The kinetic parameters for hydrolytic reaction were related to enzyme source and physicochemical characteristics of substrate. Michaelis constant \( K_m \) and maximum reaction rate \( V_{\text{max}} \) for hydrolysis of olive TAG by \( R. javanicus \) lipase were significantly higher than those by \( C. cylindracea \) lipase. The kinetic parameters for coconut TAG were lower than those for olive TAG. The \( K_m \) and \( V_{\text{max}} \) for TAG hydrolysis increased with increase in reaction temperature and decreased with increase in reaction pH. Buffer component may have considerable effect on enzyme activity and R-activity profile of lipases in reverse micelles. When considering the influence of reaction variables on lipase-catalyzed reactions for practical purposes, the initial rate as well as molar yield over extended period should be studied. The effect of a reaction condition, e.g., surfactant concentration, could be different on initial rate and degree of hydrolysis (% of TAG hydrolyzed at 24 hr). Lipase activity was rapidly reduced in reverse micellar media in absence of the substrate. The effect was severe at higher values of R. The stability of enzyme in reverse micelles was also related to the source of the enzyme. \( R. javanicus \) lipase exhibited more stability than \( C. cylindracea \) lipase did.

The four reaction parameters—pH, reaction temperature, R, and Aerosol-OT concentration—had significant linear, quadratic and interactive effects on the initial rate and the degree of hydrolysis for lipase-mediated hydrolysis of TAG in Aerosol-OT/iso-octane reverse micellar media. Reaction temperature and pH had the most significant influence on the rate and the degree of hydrolysis; whereas Aerosol-OT concentration had the least influence on those parameters. Initial rate was primarily influenced by the interactive effect of pH with all other variables; whereas degree of hydrolysis was primarily influenced by the interactive effect of reaction temperature with other variables. Lower-level variable combinations were favorable over higher-level variable combinations for TAG hydrolysis in reverse micellar media. Regression models, developed for the initial rate and the degree of hydrolysis as a function of reaction variables, accounted for up to 96% of the variation in the two responses. The optimum reaction condition ranges were determined based on maximization of both the rate and the degree of hydrolysis. The differences in the physicochemical characteristics of substrates had no significant effect on the optimum condition ranges. However, noticeable differences were
observed for these ranges between the systems with *R. javanicus* and *C. cylindracea* lipases. Lipase-catalyzed hydrolysis of TAG in Aerosol-OT/iso-octane reverse micellar media was optimum at about 22°C, 140 mM Aerosol-OT concentration, pH 6.8, and $R = 14$.

The novel solvent-free reverse micellar media composed of lecithin as a surfactant and triacylglycerols as the continuous non-polar phase is suitable for lipase-catalyzed hydrolysis of TAG. Reaction temperature, pH, and molar ratio ($R$) of water to surfactant had significant linear, quadratic, and interactive effects on the initial rate and the degree of hydrolysis for *R. javanicus* and *C. cylindracea* lipase-catalyzed hydrolysis of olive and coconut TAG. Temperature and $R$ had the most significant influence on the lipase catalysis. In contrast to Aerosol-OT/iso-octane system, lipase catalysis in solvent-free system was least sensitive to pH. Both enzymes exhibited unusually high thermal stability in this system. Regression models developed for the initial rate and the degree of hydrolysis as a function of the reaction variables accounted for up to 98% of the variation in the two responses. Optimum reaction conditions were determined based on maximization of both the rate and the degree of hydrolysis. Unlike the Aerosol-OT/iso-octane system, the optimum reaction conditions were highly dependent on the physicochemical characteristics of the substrates. The optimum reaction conditions were also dependent on the intrinsic characteristics of the enzymes. It is possible to achieve high degrees of TAG hydrolysis by maintaining or gradually increasing the $R$ value during hydrolysis by intermittent addition of water.

*R. javanicus* and *C. cylindracea* lipases exhibited considerably different activity in the three types of reaction media, namely, solvent-based reverse micelles of Aerosol-OT, solvent-free reverse micelles of lecithin, and lecithin-stabilized aqueous emulsion. The specific activity and normalized initial rate for olive TAG hydrolysis by either enzyme were higher in the two reverse micellar media as compared with the aqueous emulsion system. The degree of hydrolysis was higher in aqueous emulsion system than that in the other two reverse micellar media. However, molar yield in the latter two systems was much higher than that in the aqueous emulsion system indicating need for lower reaction volumes for two reverse micellar media. The difference in the kinetics of the reactants, intermediates, and products among the three systems was marginal. Both enzymes exhibited different positional selectivity in different media. *R. javanicus* lipase exhibited high degree of selectivity for *sn*-1 or *sn*-3 position in solvent-based and solvent-free reverse micellar media; whereas there was no specificity in aqueous emulsion. *C. cylindracea* lipase exhibited no positional specificity in solvent-based Aerosol-OT reverse micellar media, and some degree of selectivity for *sn*-2 position in solvent-free lecithin reverse micelles and aqueous emulsion. Both enzymes showed remarkable differences among the three reaction media in terms of optimum reaction conditions. Both enzymes exhibited unusually high thermal stability in the solvent-free lecithin reverse micellar medium as compared with the other two systems. The optimum $R$ value was lower in the solvent-based Aerosol-OT reverse micellar medium than that in the solvent-free lecithin reverse micellar medium. There was no apparent difference in the pH optimum in Aerosol-OT reverse micellar medium and the aqueous emulsion; however, the optimum pH in both systems was considerably higher than that in lecithin reverse micellar media.
Lipase-mediated hydrolytic reactions in the solvent-free reverse micellar media composed of natural phospholipids (soy lecithin) as the surfactant and TAG as the continuous non-polar phase can be used for the bioconversion of milk fat to value-added food ingredients (Patel et al., 1996d). The reaction parameters had significant linear, quadratic, and interactive effects on the hydrolytic reaction of \textit{R. javanicus} and \textit{C. cylindracea} lipases and on the free fatty acid (FFA) profile, content and composition of monoacylglycerols (MAG) and diacylglycerols (DAG) in milk fat hydrolysates prepared by \textit{R. javanicus} lipase. The initial reaction rate for milk fat hydrolysis was dependent on all three variables. The degree of hydrolysis was independent of the reaction pH. Both enzymes exhibited remarkably high thermal stability in this reaction system. The content and the composition of various components in milk fat hydrolysates prepared by \textit{R. javanicus} lipase were most influenced by reaction temperature. The optimum reaction conditions necessary for favoring production of short-chain FFA, MAG, DAG, and specific regio-isomers of MAG and DAG have been defined. Thus, it is possible to use this novel system for tailoring the production of milk fat hydrolysates with desired multiple functionality by selecting appropriate reaction conditions.

This study has demonstrated that the solvent-based and solvent-free reverse micellar systems could be used for lipase-mediated bioconversions of TAG to free fatty acids, glycerols, and other value-added ingredients. However, these reaction systems cannot be made economically feasible process, unless efficient protocol for product separation and enzyme recovery for reuse can be developed. Further research to develop such protocols, as described below, will be necessary for the commercialization of lipase catalysis in reverse micellar media.

\textit{Esterification/Interesterification Reactions:} Lipase-catalyzed synthesis reactions necessitate the use of low-water environments and reverse micelles have been shown to be particularly useful in this regard. Nagao and Kito (1990) discussed in detail the possibilities of lipase-catalyzed fatty esters for use in the food industry. In this article, they point out the advantages of reverse micelles in facilitating such syntheses. In general, microemulsion/reverse micelle-based interesterification and transesterification reactions are more efficient and easier to perform than reactions using immobilized enzymes.

Holmberg and Osterberg (1987) and Osterber et al. (1989) demonstrated that microemulsions composed of the anionic surfactant AOT in hydrocarbon solvents as well as non-ionic surfactants such as triethylene glycol monodecyl ether in hydrocarbon could be used for the lipase-catalyzed interesterification of triglycerides and fatty acids. The reaction rate in the non-ionic surfactant microemulsion was higher than that in the AOT reverse micelles. Moreover, addition of water to the reverse micelles composed of the non-ionic surfactant resulted in phase-splitting into an aqueous phase containing the enzyme while the surfactant was concentrated in the organic solvent phase permitting facile enzyme recovery. Similarly, Bello et al. (1987) studied the lipase-catalyzed interesterification in a quaternary system composed of triglyceride and fatty acids, water, surfactant Brij 35, and alcohol. As the water activity was reduced, interesterification was favored over hydrolysis. At optimum
LIPASE CATALYZED REACTIONS

conditions, 40% conversion could be achieved for the interesterification reaction. Abraham et al. (1988) observed that in reverse micelles composed of AOT in hexane, triacetin-tributyrin interesterification was favored over hydrolysis as the degree of hydration of the micelles was lowered. Hayes and Gulari (1990) conducted a detailed kinetic study of lipase-catalyzed interesterification in Aerosol-OT/iso-octane reversed micelles. They observed that, while the pH profile was unchanged, the ratio of water to surfactant had a profound effect on activity as well as stability of the enzyme.

Lipase-catalyzed synthesis of triacylglycerols has also been found to be favorable in reverse micelles. Morita et al. (1984) conducted the synthesis of triacylglycerol by lipase in phosphatidylcholine reverse micelles in n-hexane. Though in other media 1,2 diacylglycerol was hydrolyzed to 2-monoacylglycerol, triacylglycerol synthesis took place in reverse micelles. The initial activity of the synthesis was optimum at \( R = 10 \). Lauric, myristic, palmitic, stearic, oleic, and arachidic acids were found to be effective substrates for the synthesis of triacylglycerol. Other studies conducted by Hayes and Gulari (1990) as well as Fletcher and Parrott (1987) showed that a 60% conversion of fatty acids to triglycerides could be achieved in reverse micelles composed of Aerosol-OT/iso-octane. Legoy et al. (1987) obtained 95% synthesis of ester from heptanol and oleic acid using C. cylindracea lipase in Brij 35 microemulsions after 14 days of reaction at optimum reaction conditions. Chang and Rhee (1990) studied glycerolysis of triacylglycerols in Aerosol-OT/iso-octane reverse micellar media using Chromobacterium viscosum lipase. The glycerolysis reaction was maximum at pH 7 and 40 °C. The maximum activity was obtained at \( R \) value of 1.21.

Thus, the reverse micellar medium is particularly useful in the synthetic reaction of lipases. The microaqueous nature of the system facilitates shifting of equilibrium towards synthetic activity.

C. Specialized Reaction Systems

Recently, efforts have been made in the area of enzyme catalysis in non-polar media to develop reaction systems that not only enhance the catalytic activity of enzyme but also facilitate separation of product as well as recovery of enzyme for reuse. For lipase catalysis, the efforts are focused in three areas: microemulsion based gels or organogels, use of other phase regions in the ternary system of surfactant/solvent/water, and use of supercritical fluids.

1. Microemulsion-Based Gels

Under certain conditions, it is possible to transform reverse micellar solutions to rigid, optically transparent gel-like structures with extremely high viscosity. These gels serve to entrap biopolymers such as proteins and enzymes with retention of catalytic activity. Microemulsion-based gels have a distinct advantage over conventional reverse micelles in that they facilitate enzyme reuse and easy product separation.

Recently, Jenta et al. (1991) have conducted a detailed kinetic study of the enzymatic transesterification activity of Chromobacterium viscosum lipase in gelatin based
organogels where octyldcanoate was synthesized from decanoic acid and octanol. Concentrations of the enzyme to the extent of 0.6 mg enzyme/mL of gel could be achieved. The catalytic activity of the enzyme was essentially invariant on changing gelatin concentration and water content of gel over a wide range. Though an attempt was made to increase surface area by gel fragmentation, severe mass transfer limitations of the substrate from the gel phase to the oil continuous phase still prevailed. This dramatically reduced the catalytic efficiency of the gel-entrapped lipase. Scartazzini and Luisi (1988) recently demonstrated that addition of small amounts of water to soybean/lecithin organic solvent reverse micelles causes a dramatic increase in viscosity and the formation of a rigid, optically transparent gel matrix. Solutions of lecithin in up to 50 different solvents are amenable to gelation on the addition of small amounts of water which causes an increase in viscosity by a factor of $10^6$. Since lecithin solutions do not contain any polymeric material, the formation of such highly viscoelastic solutions is intriguing and a major research thrust has aimed at elucidating the microstructure of lecithin gels (Schurtenberger et al., 1989; Luisi et al., 1990; Capitani et al., 1991). Scartazzini and Luisi (1990) have investigated the lipase-catalyzed hydrolysis of tricaprylin using enzyme immobilized in microemulsion gels of soybean lecithin in cyclooctane. The reaction was slow and was carried out over a period of 8 days to obtain moderate yields. The enzyme was active in the gel for about a month. However, the free fatty acids produced as a result of the hydrolysis reaction acted as co-surfactant and destabilized the gel causing the viscosity to decrease appreciably. Eventually, the gel was converted to a viscous solution.

2. Various Phase Regions of Ternary System

Most studies on enzymatic reactions in microheterogeneous media have used the region on the phase diagram of surfactant/solvent/water ternary system corresponding to the formation of reverse micelles. However, it has been shown that it is also possible to entrap enzymes in surfactant aggregates where the spherical micellar structures do not prevail in phase regions such as lyotropic liquid crystal mesophase, biocontinuous microemulsion, and middle phase microemulsion (Winsor III system). These types of reaction systems utilizing phase regions other than reverse micellar system not only entrap the enzyme for catalysis but also facilitate the product separation and enzyme recovery by temperature and concentration induced phase changes. Use of Winsor III system was investigated by lipase-catalyzed hydrolysis of triacylglycerols (Sonesson and Holmberg, 1991). The system was prepared by addition of polyoxyethylene ether phosphate surfactant to equal volume mixture of buffer and iso-octane. Since lipase is a membrane protein, 95% of the enzyme was concentrated in the middle phase microemulsion. The rate of enzymatic hydrolysis of trimyristin to 2-myristoyl glycerol and fatty acid was comparable to that in Aerosol-OT/iso-octane reverse micelles. The fatty acids produced remained in the bottom phase and could be easily separated.
3. Supercritical Fluids

Supercritical fluids formed by compression of carbon dioxide has been suggested as an interesting medium for biocatalytic reactions. Such system permits high mass transfer rate and easy separation of reaction product. Recently, a few studies have explored the supercritical carbon dioxide for lipase-mediated interesterification reactions.

The interesterification of trilaurin and myristic acid, catalyzed by a 1,3-specific lipase from *Rhizopus arrhizus*, was investigated in supercritical carbon dioxide using a continuous-flow packed-bed reactor containing lipase covalently attached to glass beads (Miller et al., 1991). The reaction rate was not influenced by mass transfer limitations over the range of flow rates studied, and lipase retained full activity at 1400 psi and 35 °C for up to 80 hr. The carbon dioxide water content did not affect the intrinsic activity of the enzyme, but a higher water concentration caused a greater degree of unwanted hydrolysis. The selectivity of the reaction for interesterification over hydrolysis improved at higher pressures as the extent of hydrolysis reaction was reduced. The activity and stability of lipase in supercritical carbon dioxide were similar to those in organic liquid solvents. Chi et al. (1988) studied *R. delemar* lipase-mediated hydrolysis and interesterification using synthetic fat in supercritical carbon dioxide at 50 °C and 29.4 MPa. The time-course of interesterification was influenced by the water content as well as by the kind of reaction medium. The initial velocities of hydrolysis and interesterification were greater in supercritical carbon dioxide than in n-hexane when the water content increased. These few studies show the potential of supercritical fluids as media for biochemical reactions. Further work aimed at developing a supercritical fluid bioreactor will be necessary for the commercialization of this reaction system.

V. CONCLUSIONS

In this review, the general characteristics of lipase catalysis are described. Applications of lipase-mediated hydrolytic and synthetic reaction in various fields are briefly reviewed. Lipase-mediated bioconversion of triacylglycerols (fats and oils) and other sparingly water-soluble compounds in practically water-free media is of current interest for the production of value-added and specialty products. Recently, several kinds of novel reaction systems have been developed for lipase catalysis in organic environments. Lipases in these system offer considerable potential for the industrial catalysis of fats and oils and related chemicals, and some processes are likely to be commercialized in the near future. Technological feasibility of many of the systems has been confirmed. Also, the factors influencing the equilibrium positions in these systems are reasonably well understood, but knowledge of kinetics is still rather limited. Ultimately, economical feasibility has to be determined for the commercial success of the process.
REFERENCES


Leu., Chern

Resolution of racemic mixtures via lipase catalysis

31, 6407 (1990).

acid

 tech.,

T.R.J., Robinson, B.H.,

Jacobsen,

Kloosterman, J., van Wassenaar, P.O. and Bel, W.J. Modification of fats and oils in membrane

Kirchner. G.,


J.S. Kang. S.T. and Rhee,

Klibanov, A.M. Enzymatic catalysis in anhydrous organic solvents.

Kaimal, T.N.B. and Saroja, M. Enhancement of catalytic activity of porcine pancreatic lipase by reduc­

Kang, S.T and Rhee, 1.S. Characteristics of immobilized lipase-catalyzed hydrolysis of olive oil of high

Kaimal, T.N.B. and Saroja, M. Selective removal of linolenic acid from soybean oil by lipase-catalyzed

Kosugi, Y., Igusa, H. and Tomizuka, N. 1987. Glyceride production from high free fatty acid rice bran oil


Kwon, D.V. and Rhee,

Kroh, H.T. and Thomson, A.R. Enzyme kinetic studies using lipase immobi­

Inada, Y., Matsushirna, A., Takahashi, K. and Saito, Y. Polyethylene glycolmodified lipase soluble and

Ibrahim, C.O., Nishio, N. and Nagai, S. The role of water in the equilibrium of esterification of immobi­


Inada, Y., Takahashi, K., Yoshimoto, T., Ajima, A., Matsu­shima, A. and Saito, Y. . Application of poly­


Inada, Y., Matsushima, A., Takahashi, K. and Saito, Y. Pol­yethylene glycolmodified lipase soluble and


Itoh, T. and Ohta, T. A simple method for the preparation of optically active α-hydroxystannanes by the


Itoh, T., Ohta, T. and Sano, M. An efficient preparation of the optically active γ-hydroxystannanes using


Jacobsen, T., Olsen, J. and Allermann, K. Production, partial purification, and immunochemical characteri­


Kaimal, T.N.B. and Saroja, M. Selective removal of linolenic acid from soybean oil by lipase-catalyzed


Kaimal, T.N.B. and Saroja, M. Enhancement of catalytic activity of porcine pancreatic lipase by reduc­tive


Kang, S.T. and Rhee, J.S. Effect of water on hydrolysis of olive oil by immobilized lipase in reverse-phase


Kang, S.T. and Rhee, J.S. Characteristics of immobilized lipase-catalyzed hydrolysis of olive oil of high


Kang, S.T. and Rhee, J.S. Effect of solvents on hydrolysis of olive oil by immobilized lipase in reverse­


Kawase, M. and Tanaka, A. Effects of chemical modification of amino acid residues on the activities of


Kery, V., Trancikova, J. and Schmidli, S. Hydrolysis of olive oil by pancreatic lipase in biphasic organic


538 (1986).


Kim, T. and Chung, K. Some characteristics of palm kernel olein hydrolysis by Rhizopus arrhizus lipase


Kirchner, G., Scollar, M.P. and Kilbanov, A.M. Resolution of racemic mixtures via lipase catalysis in


Kloosterman, J.V., van Wassenaar, P.D. and Bel, W.J. Modification of fats and oils in membrane bioreac­


Kosugi, Y., Igusa, H. and Tomizuka, N. 1987. Glyceride production from high free fatty acid rice bran oil


Kwon, D.Y. and Rhee, J.S. Effect of organic solvents on lipase for interesterification of fats and oils


