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Hydrolysis of Oils by Using Immobilized Lipase Enzyme: A Review

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Abstract This review focuses on the use of immobilized lipase technology for the hydrolysis of oils. The importance of lipase catalyzed fat splitting process, the various immobilization procedures, kinetics, deactivation kinetics, New immobilized lipases for chiral resolution, reactor configurations, and process considerations are all reviewed and discussed.

Keywords: hydrolysis, enzyme immobilization, enzyme kinetics, deactivation kinetics, immobilized enzyme reactors, chiral compounds

IMPORTANCE OF LIPASE-CATALYZED FAT SPLITTING PROCESS

The production of fatty acids by the hydrolysis of natural oils and fats is a very important component in the economic exploitation of these naturally produced renewable raw materials. These products include oils from corn, rapeseed, sunflower, palm, coconut, olives and rice bran, and a wide range of animal fats such as tallow's. A significant number of high-value products require fatty acids in their manufactures. These include coatings, adhesives, specially lubricating oils, shampoos and other personal care products.

Oils and fats are part of a group of compounds known as fatty esters or triglycerides, and their hydrolysis essentially involves reactions with water to produce valuable free fatty acids and glycerol. There are three major routes currently used for the hydrolysis of fats and oils in the production of fatty acids; high pressure steam splitting, alkaline hydrolysis and enzymatic hydrolysis. The high temperature and pressure (typically 250°C, 70 bar) necessary for steam splitting make this process unsuitable for splitting sensitive triglycosides, unconjugated systems (which may undergo thermal degradation), hydroxylated fats and oils (which may dehydrate) or polyunsaturated oils with high iodine number (which may polymerize). There are also difficulties associated with alkaline hydrolysis, namely high energy costs and the need to acidify the soaps formed, to produce the fatty acid products.

Enzymatic hydrolysis of triglycerides may be carried out at ambient conditions (typically 35°C and atmospheric pressure), making it energy efficient in comparison to the steam splitting process. In this technology an aqueous solution of lipase is contacted with the oil, forming a liquid –liquid dispersion. The lipases are enzymes that specifically catalyze the hydrolysis of the oils into free fatty acids and glycerol at the interface between the two liquids. The hydrolysis reaction yields 1mole of glycerol and 3moles of fatty acids per mole of triglycerides. Triglycerides here on called "lipids", do not dissolve in the water phase, so the reaction has to take place at the interface of the water and lipid phase [1]. The products, fatty acids and glycerol, are dissolve in the lipid water phase respectively. As the reaction is reversible both the hydrolysis rate and the final composition depends on the fatty acid concentration in the oil phase and on the glycerol concentration in the water phase.

The hydrolysis of oils and fats is an important industrial operation: world wide 1.6×10^6 tons of fatty acids are produced every year by this process [1]. Some factories in Japan already use lipase for production of soap powder and production of high purity unsaturated fatty acids [2]. However the enzyme splitting process is not yet widely used because the time for lipolysis of a single batch is 2-3 days, continuous processing is difficult, and the price of enzyme is high. For the sake of energy conservation and minimizing thermal degradation of the products, the drawbacks of this process, researchers have set to study the hydrolysis of fats and oils catalyzed by the lipase enzyme. One of the intrinsic features of this surface-active enzyme is its activation by interfaces. Therefore, in order to increase the interfacial area of the oil-in-water emulsion, emulsifiers have been added [3]. Yet the reproducibility of kinetic run with an emulsion is particularly difficult to obtain because the stability of an emulsion is low and strongly dependent on the method of preparation.

ENZYME IMMOBILIZATION

Immobilized means it has been confined or localized so that it can be reused continuously. The first attempt to immobilize a biocatalyst back to 1953, while in 1969

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an immobilized enzyme was used for the first time in an industrial process, since then this technique has gained more and more importance, and now a wide variety of immobilized enzymes are employed in the food, pharmaceutical and chemical industries. Although lipases presently account for no more than 3% of all enzymes produced worldwide, the use of immobilized lipases for the modification of melted fats and oils is currently a subject of expanding interest. This interest is due in part to the fact that the use of lipases has the potential to be more cost effective when enzymes are employed in immobilized rather than in free form. In principle immobilized lipase technology would facilitate the development of continuous, large-scale commercial processes (as opposed to most small scale, batch operations that employ soluble lipases for the production of food flavors) which have a high efficiency per unit volume of reactor corresponding high rate of return of capital costs [4]. Further more, the use of immobilized lipases leads to a decrease in potential for contamination of the product via residual lipases, thus avoiding the need for down stream thermal treatment. Immobilization also permits multiple uses of the lipases and often enhances its thermal and chemical stability, thus leading to predictable decay rates. It also enhances opportunities for better control of both the process and product quality. In fact the incremental costs of using an immobilized biocatalyst in a continuous process are more than 20 times lower than with a traditional one, arising primarily from the cost of the relatively large amount of non-reusable enzyme required by the latter process. The main disadvantages of immobilization can be, loss of activity due to immobilization, limitation in substrate's diffusion, possible leakage of the biocatalyst from the support.

METHODS OF IMMOBILIZATION

Numerous methods for achieving the immobilization of lipases are available; each involves a different degree of complexity and efficiency. The various methods used to date are: adsorption [3-10], ionic bonding, covalent binding [11], cross-linking [12], entrapment, and encapsulation [13].

Adsorption

It is the simplest method and involves reversible surface interactions between enzymes and support material. The forces involved are mostly hydrophobic. Among the advantages are: it is a cheap, fast and simple process; no chemical changes to support or enzymes are necessary; it is a reversible immobilization. Disadvantages are the leakage of the enzyme from the support, the possible steric hindrance by the support and the nonspecific binding. Inorganic (activated carbon, silica, *etc*.) or organic (natural or synthetic polymers) compounds can be used as supports.

Ionic Binding

It is based on electrostatic interactions (ionic and hydrogen bonding) between differently charged ionic groups of the matrix and of the enzymes. The advantages and disadvantages are the same of the adsorption process, but through ionic binding the enzyme conformation is influenced more than through adsorption and less than through covalent binding. Different anion exchange materials, like DEAE-cellulose or DEAE-Sephadex are used as matrices.

Covalent Binding

This method is based on the formation of covalent bonds between a support material and some functional groups of the amino acid residues on the surface of the enzyme. Usually, the support has to be first activated by a specific reagent, to make its functional groups strongly electrophilic; these groups are then allowed to react with strong nucleophilic groups of the enzyme. The advantage of this method is the strength of the bond and the consequent stability of immobilization; the disadvantages are the higher costs and the lower yields, as the enzyme conformation and of course activity will be strongly influenced by the covalent binding.

Cross-linking

This type of immobilization is support –free and involves joining the enzymes to each other to form a three-dimensional structure. The bond is formed by means of a bi-or multifunctional reagent such as glutaraldyhyde. The disadvantages are the very low immobilization yields, the absence of mechanical properties and the poor stability.

Entrapment

Using this method, the enzyme is free in solution, but restricted in movement by the lattice structure of a gel. There are different methods for the entrapment such as temperature-induced gelation, polymerization by chemical/photochemical reaction, ionotropic gelation of macromolecules with multivalent cations. Entrapment is mainly used for immobilization of cells, but has the inevitable disadvantage that the support will act as a barrier to mass transfer.

Encapsulation

Encapsulation of enzymes or cells can be achieved by enveloping the biological components within various forms of semi permeable membranes, usually microcapsules varying from 10-100 μm in diameter. Large proteins cannot pass out of or into the capsule, but small substrates and products can pass freely across the semi permeable membrane. A disadvantage is the acute diffusion problem; an advantage can be the co-immobilization of different enzymes/cells to suit particular ap-

plications.

The selection of an immobilization strategy is based on process specifications for the catalyst, including such parameters as overall enzymatic activity, effectiveness of lipase utilization, deactivation and regeneration characteristics, cost of the immobilization procedure, toxicity of immobilization reagents and the final properties of the immobilized lipase [4].

KINETICS OF THE ENZYMATIC REACTION

Several mechanisms have been proposed for lipase catalysis reactions [14]. The vast majorities of these mechanisms were developed for the case of soluble lipases acting on insoluble substrates (*e.g.* oil droplets dispersed in water). However, in the absence of diffusional limitations the validity of the aforementioned mechanism easily extended to include the most complex case of having the lipases present in immobilized states. The simplest kinetic model applied to describe lipase-catalyzed reaction is based on the classic Michaelis-Menten mechanism as applied to emulsified oil/water systems [15]. The kinetic steps can be represented schematically as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E+P+Q \quad (1)$$

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

$$r_v = V_{max}[S]/K_m + [S] \quad (2)$$

$$V_{max} = k_{cat}\,[E]_{tot} \quad (3)$$

$$K_m = k_{cat} + k_{-1}/k_1 \quad (4)$$

Where V_{max} is the rate observed when the lipase is saturated with substrate, K_m is the Michaelis-Menten constant, the bracket denote molar concentrations of the various species, and the subscript tot denotes the total amount.

In the case of feed stocks from natural origin which contain more one chemical species susceptible to lipase action (*e.g.*, butter fat), the Michaelis-Menten mechanism denoted as equation (2) may be extended in order to include competitive inhibition by every substrate, S_i with respect to each other. For extent of hydrolysis below 70%, a pseudo-zero order rate expression arises which takes the form

$$r_{vi} = \frac{V_{max,i}\,[S_i]}{\sum_{j=1}^{N}[S_i]} \quad (5)$$

$$V_{max,i} = k_{cat,i}[E]_{tot} \quad (6)$$

The above equation is based on the following assumptions: (1) the Michaelis-Menten constants, K_{mi}, associated with every substrate S_i (out of N possible substrates) are approximately equal; and (2) all $[S_i]/K_{mi}$ are very large compared to unity.

Three rate expressions were utilized based on Michaelis-Menten kinetics and a ping-pong bi-bi mechanism to fit the uniresponse experimental data for the total rate of release of fatty acids.

$$\text{Model A: } r_{V,hyd,A} = \theta_{A,1}[G] \quad (7)$$

$$\text{Model B: } r_{V,hyd,B} = \frac{\theta_{B,1}[G]}{1+\theta_{B,2}[G]} \quad (8)$$

$$\text{Model C: } r_{V,hyd,C} = \frac{\theta_{C,1}[G]}{1+\theta_{C,2}[G]+\theta_{C,3}[G]^2} \quad (9)$$

where $r_{V,hyd,A}$, $r_{V,hyd,B}$, and $r_{V,hyd,C}$ correspond to the effective rate of reaction per unit volume (M/h) for the various models, [G] is the concentration (M) of glyceride moieties (accessible ester bonds, and θ_i represent lumped parameter related to the rate parameter appearing in the various Michaelis-Menten models (V_{max} parameter, and Michaelis and inhibition constant). These rate expressions are identical in mathematical form to those employed by Malcata *et al.* [16,17]. If the feedstock does not contain, initially, significant amount of free fatty acids, then [G] can be determined by the following equation.

$$[G] = [G_0] - [FA] \quad (10)$$

Where $[G_0]$ represents the initial concentration of glyceride bonds accessible to hydrolysis and [*FA*] is the concentration of free fatty acids resulting from hydrolysis.

Deactivation Kinetics

One of disadvantages, and probably the most serious disadvantage, of biocatalyst is that they lose their catalytic activities during the reaction. This phenomenon is well recognized in biotechnology and is called decay, inactivation, or denaturation of the biocatalyst. Even if the life of a biocatalyst can be prolonged by its immobilization, it still loses its activity sooner or later. The catalytic durability of the biocatalyst during continuous operation is called operation stability. The operational stability of a biocatalyst is estimated by its half-life, which is the elapsed time at which the catalytic activity is reduced to half. The half-life is a very important parameter that governs the economical feasibility of the bioprocess concerned [18].

Two methods are known to evaluate the deactivating catalyst during continuous operation. For the most common case continuous operation is performed so as to keep the conversion fixed (constant conversion). This requires decreasing flow rate as the enzyme activity drops gradually. In other method the flow rates (or the

space velocity) is kept constant throughout the continuous operation (constant feed rate or constant space velocity) to observe the decrease in the outlet conversion. The former method requires a precise feed back control strategy. Otherwise, trial and error in changing the flow rate is inevitable as the exact profile of the activity decay is unknown prior to the experiment. Because of the ease of experimentation, the later policy has been often applied in the biotechnological area. Generally, deactivation rates are determined in the absence of substrate, but enzyme deactivation rates can be considerably modified by the presence of substrate and other materials.

Biocatalyst thermal stability is a fundamental aspect in the reactor performance. Despite this, most information on biocatalyst stability, being gathered under non-reactive conditions, is of limited use, leaving aside modulation effects by substrate and products, which certainly play a role during catalysis. Only in few cases, the modulation of enzyme inactivation by reagents and products has been studied and made explicit in reactor modeling [19-22].

Different mechanisms have been proposed to describe enzyme thermal inactivation. The simplest and most used is one stage first-order kinetics, which proposes the transition of a fully active native enzyme to a fully inactivated species in a single step. Such mechanism leads to a model of exponential decay:

$$\frac{e}{e_o} = exp(-k_D t) \tag{11}$$

Thermal inactivation is certainly more complex and series and parallel mechanisms have been proposed to describe it [23]. Models derived from such mechanisms contain a high number of parameters, which are difficult to determine experimentally. However, a two-phase series mechanism usually represents well the phenomenon in terms of a limited number of parameters susceptible to reliable experimental determination. A model based on such mechanism is represented by equation (12):

$$\frac{e}{e_o} = \left[1 + A\frac{k_1}{k_1 - k_2}\right] exp(-k_1 t) - A\frac{k_1}{k_1 - k_2} exp(-k_2 t) \tag{12}$$

Where e stands for enzyme activity, e_o is its initial value, t stands for time, k_1 and k_2 are the transition rate constants in each inactivation stage and A is the specific activity ratio between the intermediate and initial enzyme stage. These models have been traditionally used to evaluate enzyme stability under non-reactive conditions [24], so that parameters obtained do not reflect the behavior in the presence of substrate and products as it occurs in the reactor. It has been postulated that any substrate that interacts with the enzyme during catalysis is a potential modulator of enzyme stability [25].

Therefore

$$-d[E_i J]/dt = k_{iJ}[E_i J] \tag{13}$$

$k_{iJ} = k_i(1-n_{iJ})$ where n_{iJ} represents the modulation factor of modulator J in inactivation stage i. For instance, in the case of an enzyme subjected to product competitive inhibition, three enzyme species will exist : the free enzyme (E) and the secondary enzyme-substrate (ES) and enzyme-product (EP) complexes, among which the enzyme will be distributed during the course of catalysis.

IMMOBILIZED LIPASES FOR CHIRAL RESOLUTION

The chemical industry has been using enzymes for biotransformation of molecules for many years. Recently, substances having "chirality" have become of great significance to the pharmaceutical industry, and enzymes are important for manipulating this characteristic.

Importance of Chirality

Synthetic compounds that exhibit chirality are usually found to have almost equal amounts of both the forms of the molecules (isomers), *i.e.* they are found as a mixture. However, it is often found that only one isomer has the desired properties of the compound, whilst the other isomer may be inactive or sometimes harmful. Thalidomide is an example of such a molecule having a desirable and effective isomer and a seriously harmful isomer. Safer drugs are required and expected.

What Are Chiral Compounds?

- The difference in the activity of the two isomers of a chiral compound derive from the following characteristics:
- 4 arms (bonds) of the carbon atom connect to different elements or chemical groups.
- This gives “left and right handed” structures (mirror images).
- Both isomers are chemically identical

Why Different Bioactivity?

Although chemically identical, and often very difficult to separate by chemical means, the two different spatial forms (optical isomers) of a chiral molecule may be recognized differently in a living organism. It is very important to be able to prepare the individual isomers in a pure form so that only the beneficial properties are expressed. This is why the production of "chiral compounds" is so important in leading to new and safer pharmaceuticals.

How Can Compounds Are Resolved?

1. The two forms of the molecules are called:
   “Optical isomers” or “Enantiomers”
2. An equimolar mixture of the two is called:

"Racemic mixture" or "Racemate"
3. Separation is called "Resolution":

Use immobilized lipase to prepare optically pure Enantiomers because the enzyme modifies only one of them and facilitates separation.

Various new lipase enzyme products for chiral synthesis in various fields such as for biotransformation use, pharmaceutical use, diagnostic use, and food processing use are listed in Tables 1-4.

IMMOBILIZED LIPASE REACTORS

The use of immobilized lipases for the hydrolysis of oils is currently a subject of expanding interest. This interest is partly due to the fact that the use of lipases is more cost effective when these enzymes are employed in immobilized rather than in free form. In general, the multiphase reactors considered for this application contain a solid phase that can be either mobile or stationary [26].

Several reactor configurations have been used in studies of immobilized lipases. Two types of phases are invariably a solid phase (*i.e.*, the carrier on which the lipase is immobilized), and one or two liquid phases (*i.e*., feedstocks). If an organic solvent is used to dissolve the reactant and product species, then only a single-liquid phase is present in the reactor. The solid phase in two-phase reactors and one of the liquid phases in three phase reactors may appear as a dispersed phase or a continuous phase. A typical configuration of the continuous solid phase corresponds to a membrane in either flat sheet or hollow fiber form. Dispersed solid phases involve the use of powders as supports. Whether the organic or the aqueous phase will constitute the dispersed phase in the three phase reactors often depends on the relative amounts of the two liquid phases. Tables 5-8 consist of a compilation of the major characteristics of the lipase reactors, which have been described in the literature.

Lipases act upon substrates that are generally much more viscous than water at the room temperature. Hence, one way to overcome the difficulties associated with operating immobilized lipase reactors for processing these substrates is by the use of suitable solvents. However, the single most important criterion in selecting a non-aqueous solvents is its compatibility with the maintenance of the catalytic activity and substrate specificity of the lipases. Solvents used to carry out reactions catalyzed by immobilized lipases include benzene, toluene, *n*-hexane, *n*-heptane, octane, iso-octane, di-isopropyl ether, petroleum ether, *etc*.

Membrane Reactors

Membrane, or diaphragm, reactors have employed in the presence of one [13] and two liquid phases [4]. In one type of reactor, organic and aqueous phases are separated by a solid membrane in which the lipase is immobilized. Two types of flow patterns have been employed: flow tangential to and flow normal to the membrane. For the tangential flow case, both the phases flow parallel to the membrane in either co-current or counter current fashion [1,4]. The pressure drop along the reactor coordinate is small, and no bulk flow through the membrane is allowed. For flow normal to the membrane one of the phases is pumped through the

Table 1. For biotransformation use

Application	Product	Form	Opt. pH	Opt. Temp	Specification
Chiral synthesis	Lipase AK"Amano" (*Pseudomonas fluorescens*)	Powder	8.0	55°C	20,000 U/g (pH 7.0)
Chiral synthesis	Lipase AYS"Amano" (*Candida rugosa*)	Powder	7.0	45°C	30,000 U/g (pH 7.0)
Chiral synthesis	Lipase AS "Amano" (*Aspergillus niger*)	Powder	6.5	45°C	13,500 U/g (pH 6.5)
Chiral synthesis	Lipase PS "Amano" (*Pseudomonas cepacia*)	Powder	7.0	50°C	30,000 U/g (pH 7.0)
Chiral synthesis	Lipase PS "Amano" (*Pseudomonas cepacia*)	Immobilized on ceramic particles	7.0	50°C	30,000 U/g (pH 7.0)
Chiral synthesis	Lipase PS "Amano" (*Pseudomonas cepacia*)	Immobilized on diatomaceous earth	7.0	50°C	8,000 U/g (pH 7.0)

Table 2. For pharmaceutical use

Application	product	Form	Opt. pH	Stable pH	Specification
Digestive preparation	New lase (*Rhizopus niveus*)	Powder	6.5-7.5	4.0-8.0	3500 U/g (pH 7.0)
Digestive preparation	Lipase AP6 (*Aspergillus niger*)	Powder	5.0-7.0	2.0-10.0	7000 U/g (pH 6.5)

Table 3. For diagnostic use

Application	Product	Form	Opt. pH	Opt. Temp	Specification
Triglyceride determination	Lipoprotein lipase "Amano"3 (*Pseudomonas* sp.)	Powder	7.0	50°C	1,000 U/mg (pH 6.0)
Triglyceride determination	Lipoprotein lipase "Amano"6 (Microorganism)	Powder	7.0	50°C	500 U/mg (pH 6.0)

Table 4. For food processing use

Application	Product	Form	Opt. pH	Opt. Temp	Specification
Dairy processing	Lipase A"Amano"6 (*Aspergillus niger*)	Powder	6.5	45°C	60,000 U/g (pH 6.0)
Dairy processing	Lipase A"Amano"10 (*Mucor javanicus*)	Powder	7.0	40°C	10,000 U/g (pH 7.0)
Dairy processing	Lipase F-AP 15 (*Rhizopus oryzae*)	Powder	7.0	40°C	150,000 U/g (pH 7.0)
Dairy processing	Lipase AY "Amano"30 (*Candida rugosa*)	Powder	7.0	45°C	30,000 U/g (pH 7.0)
Dairy processing	Lipase G"Amano"50 (*Penicillium camerbertii*)	Powder	5.0	45°C	50,000 U/g (pH5.6)
Fats&oils processing	Lipase A"Amano" 6 (*Aspergillus niger*)	Powder	6.5	45°C	60,000 U/g (pH 6.0)
Fats&oils processing	LipaseM"Amano"10 (*Mucor javanicus*))	Powder	7.0	40°C	10,000 U/g (pH 7.0)
Fats&oils processing	LipaseG"Amano" 50 (*Pencillium camembertii*)	Powder	5.0	45°C	50,000 U/g (pH 5.6)
Fats&oils processing	Lipase F-AP15 (*Rhizopus oryzae*)	Powder	7.0	40°C	150,000 U/g (pH 7.0)
Fats&oils processing	Lipase AY "Amano" 30 (*Candida rugosa*)	Powder	7.0	45°C	30,000 U/g (pH 7.0)
Fats&oils processing	Newlase F (*Rhizopus niveus*)	Powder	7.0	40°C	30,000 U/g (pH 7.0)

Table 5. Reactors using a membrane support for hydrolysis of oils

Type of reactor	Source of lipase	Method of binding	Support	Purpose
Tangential flow membrane	*Candida cylidracea*	Adsorption	Polypropylene	Hydrolysis of olive oil
Flow-thru membrane	*Candida cylidracea*	Adsorption	Polypropelene	Hydrolysis of oil
Hollow-fiber	*Candida rugosa*	Adsorption	Cellulose	Hydrolysis of soyabean oil
Flow-thru membrane	*Aspergillus niger*	Adsorption	Microporous polypropylene	Hydrolysis of butter fat
Flow-thru membrane	*Aspergillus niger*	Adsorption	Microporous polypropylene	Hydrolysis of butter fat
Ultra filtration ceramic membrane	*Chromobacterium viscosum*	Encapsulation	Reversed micelles of dioctyl sodium sulfosuccinate in Iso-octane	Hydrolysis of olive oil
Hollow-fiber	*Candida cylindracea*	Adsorption	Microporous polypropelene	Hydrolysis of Menhaden oil

Table 6. Packed bed reactors used for hydrolysis of oils

Source of lipase	Method of binding	Support	Purpose
Aspergillus niger	Adsorption	Porous glass	Oil hydrolysis
Rhizopus arrhizus	Cell binding	Fungal mycelia	Oil hydrolysis
Candida cylindracea	Adsorption	Spherosil	Oil hydrolysis
Candida cylidracea	Adsorption	HDPE	Oil hydrolysis
Pseudomonas fluorescens	Adsorption	Dowex	Hydrolysis of beef Tallow
Candida rugosa	Adsorption	DEAE-Sephadex, Sephadex G50, Amberlite IRA94	Hydrolysis of olive oil
Candida lipolytica	Adsorption	Alumina beads	Hydrolysis of rice bran oil

Table 7. Stirred continuous Reactors using an immobilized lipase for hydrolysis of oils

Type of reactor	Source of lipase	Method of binding	Support	Purpose
CSTR	*Candida cylindracea*	Adsorption	Celite,cellulose,ethyl cellulose, silicagel,clay, kiesselguhr, alumina, CPG, carbon, nylon, polypropylene, HDPE	Hydrolysis of olive oil
Fluidized bed	*Pseudomonas fluorescens*	Adsorption	Dowex	Hydrolysis of olive oil

Table 8. Stirred batch reactors used for hydrolysis of various oils and fats

Source of lipase	Method of binding	Support
Pseudomonas mephitica	Cell binding	Bacterial cell debris
Aspergillus niger, pseudomonas fluorescens, Rhizopus japonica, R.delemar, Candida cylindracea, Hog pancreas	Covalent, Adsorption	Porous glass, sepharose, Dowex
Rhizopus arrhizus	Cell binding	Fungal mycelia
Candida cylindracea	Entrapment, adsorption, covalent	Duolite, Celite, CPG, Spherosil, Polyurethan
Candida rugosa	Adsorption	Hollow fiber membrane
Cadida rugosa	Entrapment	Polyethylene glycol, polypropylene glycol
Candida rugosa	Adsorption	Sephadex LH-20
Candida rugosa	Adsorption	Sephadex
Candida lipolytica	Adsorption	Alumina beads
Candida lipolytica	Adsorption	Alumina beads

membrane, where as the other phases remain stationary, one thus forms a coarse mixture of water and finely divided oil droplets (or vice versa) which is readily separated by gravity settling.

Packed Reactors

The fixed bed reactor has traditionally been used for most large scale catalytic reactions because of its high-efficiency, low cost, and ease of construction and operation. The packed bed reactor usually provides more surface area for reaction per unit volume than does a membrane reactor. The packed bed configuration has been employed for lipase-catalyzed hydrolysis by a number of investigators [2,6]. Granules of various sizes with a lipase previously attached to them or lipase concontaining dried mycelia are usually confined in a jacket column. If only a single liquid phase is employed, this phase may be pumped upward in order to reduce the tendency for bypassing of fluid or downward in order to take advantage of the driving force of gravity. In the case of multi-phase liquid systems, the two streams may flow through the reactor in opposite direction with denser phase flowing downwards or in the same direction.

There are two operational constraints which must be considered when operating packed bed reactor: 1) intra particle diffusion limitations on reaction rates; 2) high pressure drop across the reactor packing.

External transport limitations are generally reduced in packed bed reactors by increasing the flow rates of the substrate through the column and changing the column reactor height-to-diameter ratio, there by increasing the linear velocity. O'Neill *et al*. [27] observed kinetic constants and showed that low efficiency of a packed bed enzyme reactor was the result of poor mass transfer due to the inability of the reactor liquid to completely permeate the support structure of the packed bed at low flow rates. Under these conditions, the immobilized enzymes are not fully utilized for the reaction.

Small particles are advantages in reducing the effect

of internal diffusion, but they increase the magnitude of any pressure drop. Regular-shaped particles are much better at reducing the problem of high-pressure drop in packed beds and of irregular flow pattern, as observed by O'Neill *et al.* [27]. In general, the size of the particle, which shows essentially no internal diffusional limitations, is so small that the pressure drops incurred become prohibitive.

Based on the pseudo-steady state assumption the design equation for immobilized packed bed reactor can be expressed as follows:

$$\frac{V}{F} = -\frac{1}{1-\varepsilon}\int_{S_{in}}^{S_{out}} \frac{dS}{\eta(-r_s)} \tag{14}$$

The volumetric rate ($-r_s$) can be separated into two terms for any enzymatic reaction whether it is one-substrate irreversible or multi substrate reversible [18].

$$(-r_s) = Ef(S,P) \tag{15}$$

the simplest expression of $f(S,P)$ is a hyperbolic function of S as typically shown by the Michaelis-Menten equation.

$$f(S) = kS/K_m + S \tag{16}$$

Equation (16) has two extremities: (i) first order kinetics, $(-r_s) = (kE/K_m)S$. where $S << K_m$ (ii) zero-order kinetics $(-r_s) = kE$ when $S >> K_m$.

$$X = (S_{in}-S_{out})/S_{in} \tag{17}$$

Equation (14) is modified into following forms, depending on the intra particle and film diffusion.

(a) No limitation of diffusion (η=1.0): when the reaction rate of the immobilized enzyme particle is not influenced by intraparticle and boundary layer diffusion, eqn(14) for packed bed reactor becomes

$$\frac{V}{F} = -\frac{1}{1-\varepsilon}\int_{S_{in}}^{S_{out}} \frac{dS}{(-r_s)} \tag{18}$$

(b) Diffusion-influenced first-order kinetics (η<1.0): The overall rate of reaction of the immobilized enzyme particle is influenced by both intraparticle diffusion and film diffusion around the particles. The effectiveness factor η of the first-order reaction in a spherical particle is given by

$$\frac{1}{\eta} = \frac{\varphi_1^2}{3(\varphi_1 coth\varphi_1 - 1)} \tag{19}$$

where φ_1 is the thiele modulus of the first-order kinetics of the sphere. The φ_1 corresponds to $(-r_s) = (kE/K_m)S$ is given by

$$\varphi_1^2 = kER^2/K_m De \tag{20}$$

Where, η is independent of S for the first-order reaction. In packed bed reactor Equation (14) becomes

$$\frac{V}{F} = \frac{R^2}{(1-\varepsilon)De}\cdot\frac{1}{3[\varphi_1 coth\varphi_1 - 1]}\frac{\ln[1-x]}{} \tag{21}$$

Continuous Stirred Tank Reactors (CSTR'S)

The different CSTR configurations have been employed by a number investigators [2]. CSTR'S posses some particular advantages over fixed bed reactors, *e.g.*, lower construction costs and efficient stirring that eliminates the presence of concentration and/or temperature gradient. In general, however, a CSTR must be larger than a packed bed reactor to achieve the same extent of reaction. In order to prevent the immobilized lipase from leaving the CSTR, a microfilter or a screen must be provided at the reactor outlet.

Based on the pseudo-steady state assumption the design equation for CSTR can be expressed as follows:

$$\frac{V}{F} = \frac{S_{in}.x}{(1-\varepsilon)\eta(-r_s)} \tag{22}$$

Relations for x and $(-r_s)$ are discussed in packed bed reactor. Depending on the intra particle and film diffusion Equation (22) is modified into following forms.

(a) No limitation of diffusion (η=1.0): When the reaction rate of the immobilized enzyme particle is not influenced by intraparticle and boundary layer diffusion, then the design equation for CSTR becomes

$$\frac{V}{F} = \frac{S_{in}.x}{(1-\varepsilon)(-r_s)}$$

(b) Diffusion-influenced first-order kinetics (η<1.0): The overall rate of reaction of the immobilized enzyme particle is influenced by both intraparticle diffusion and film diffusion around the particles. The effectiveness factor (η) and thiele modulus of first order reaction (φ_1) of a spherical particle are given by Equations (19) and (20). Then the CSTR design equation becomes

$$\frac{V}{F} = \frac{R^2}{(1-\varepsilon)De}\cdot\frac{1}{3[\varphi_1 coth\varphi_1 - 1]}\cdot\frac{x}{(1-x)}$$

Fluidized Bed Reactors

Fluidized bed reactors also have been employed to effect the hydrolysis of triglycerides. For example, a fluidized bed reactor has been used for continuous hydrolysis of olive oil by Kosugu *et al*. [2]. This type of reactor has several advantages over a fixed bed reactor, namely, lower pressure drop at high flow rates, less channeling, reduced coalescence of the emulsion particles, freedom from plugging by feed particulates, and ease of simulation due to the absence of concentration gradients.

Batch Reactors

The stirred batch reactor is the type of reactor most commonly employed in bench scale and industrial scale applications [1,5]. Batch reactor are extremely versatile and easy to operate. Configuration include glass flasks stirred with magnetic bars and vessels stirred by submerged impellers. In the case of the well-stirred batch reactor, sampling can be accomplished at a single, arbitrary located point. In emulsion, the most common feed stock configuration for hydrolysis, lipases immobilized on powdered supports are very likely to collide with suspended oil droplets.

PROCESS CONSIDERATIONS

When assessing the potential of a new technology in a manufacturing plant, one must address two questions: (1) whether the process can produce the desired end product in good yield and at the desired degree of purity (technical feasibility); and (2) whether the process can produce the product at a reasonable cost (commercial feasibility). The former can be demonstrated in small systems on a bench scale, where as assessment of the latter question deals with economical considerations involving both capital costs and operational costs as well as the market for the product(s).

Enzyme deactivation plays a significant role in biological processes. Most literature on enzyme kinetics is devoted to initial rate data and analysis of the reversible effects on enzyme activity. In many applications and process settings, however, the rate at which the enzyme activity declines is of critical importance. This is especially true when considering it's long-term use in continuous reactors. In such situations the economical feasibility of the process may hinge on the useful life time of the enzyme biocatalyst.

Benefits of a solvent-free system over a solvent-based system should be balanced against its disadvantages. For the practical applications, the solvent should be dealt with cautiously because of the safety problems, and loss of solvent caused by the evaporation should be minimized. Also, the cost for separating the solvent from the product should be considered. Solvent –free system offer several advantages, including greater safety, reduction in solvent extraction costs, increase in reactant concentration in the reactor and consequent reduction in mass transfer limitations. These factors must be weighed against the effect on the catalyst activity caused by the increased solubility of water in the reactants and the increased pumping cost associated with use of the various reactant mixture.

Some of the major obstacles to the use of immobilized enzyme bio reactors with in oleochemical industry arise because the number of high added value products are limited, the enzymes involved in the bio transformations of the lipids are costly; and there are many difficulties in solving the engineering problems because of heterogeneous and/or micro aqueous nature of biochemical reactions [18]. However, increased cost of energy obtained from fossil fuels and increased demand for higher quality products coupled with vary narrow purity specifications are likely to lead to new incentives for the biochemical transformation of fats and oils. Hence, immobilized lipase processes offer great potential for future development.

NOMENCLATURE

CPG	Controlled pore glass
CSTR	Continuous flow stirred tank reactor
HDPE	High-density polyethylene
D_e	Effective diffusivity (m^2/s)
E	Enzyme concentration for immobilized enzyme (u/m^3) [one unit (U) of Lipase will liberate one micro mole of free fatty acid in one minute under assay conditions]
$f(S)$,$f(S,P)$	functions of S and functions of S and P.
F	Volumetric feed rate of substrate solution (m^3/s)
F/V	Space velocity (s^{-1})
k	Rate constant of decomposition of ES complex (s^{-1})
K_m	Michaelis constant (mol/L)
r_s	Volumetric reaction rate (mol/L/S)
R	Radius of spherical particle (m)
S	Substrate concentration (mol/L)
S_{in} , S_{out}	Inlet and out let substrate concentrations (mol/L)
t	time (s)
V	Working volume of bioreactor (m^3)
x	Conversion $=1- S_{out}/S_{in}$ (dimensionless)

Greek Letters

ε	Void fraction (dimensionless)
η	Effectiveness factor of immobilized enzyme particle (dimensionless)
φ_1	$R(kE/K_mD_e)^{1/2}$ Thiele modulus of first-order reaction in sphere (dimensionless)

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