OLÉAGINEUX, CORPS GRAS, LIPIDES

LIPOCHIMIE

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Summary

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Article

Introduction

The obvious question is why researchers have not relied on the sorcerer's apprentice to do this difficult job, but seriously, let us get right to the heart of matter, as magic is unlikely to be of much help to modern researchers.

Before talking about how to customize a process or an enzyme to reach optimum conditions of reactions, we have to recall some principles to avoid to develop customizing techniques which could inhibit or modify the catalytic activity of a given enzyme because one or several basic principles would have been ignored.

Lypase paradigm

It is now well known that the active site of acyltransferases, including lipases, is a triad of amino-acids, more or less buried in the proteic network.

Whether the famous amphiphilic lid is present or not, the triad must be accessible to the substrate to allow the enzyme to catalyze nucleophilic substitutions like esters hydrolyses or syntheses.

In lipases the triad is formed with serine, histidine and aspartic or glutamic acid.

Hydrolysis and esterification mechanism

In *figure 1* we are presenting the mechanism of hydrolysis admitted today and described by Verger and Schmid [1]. The hydroxyl group of serine is first activated by a succession of electric charges shifts which affects *in fine* the triad in the so-called oxyanionic hole, providing a very powerful nucleophic reactivity to the hydroxyl group of serine.

One of the two available electron pairs of the oxygen, attacks the carbon Sp2 of the ester bond of the substrate to form an oxyanionic tetrahedral intermediate which is stabilized through hydrogen bonds with peptidic NH groups.

The return of the electron pair of the negative charge, to form again the CO double bond, leads to the release of the alkoxy moiety which withdraws the proton of the imidazolium ion to form a free alcohol immediately ejected out of the catalytic hole.

The acyl chain stays linked through a covalent bond with serine to form the acylenzyme.

This intermediate is then attacked by a molecule of water according to the same nucleophilic substitution mechanism leading to a second tetrahedral intermediate which breaks down to release the carboxylic acid; the enzyme recovers at the same time its initial state.

In the case of esters synthesis, carboxylic acids bind readily to serine hydroxyl releasing a molecule of water which is immediately trapped by hydrogen bonds in the proteic network *(figure 2)*. The acylenzyme is then attacked by a molecule of alcohol playing the role of the nucleophilic reagent through one of the two electron

pairs of the oxygen atom; the reaction gives rise to an ester molecule.

In the case of acyl exchanges between different esters namely interesterification, the reaction proceeds through a succession of hydrolyses and resyntheses.

Accordingly apart hydrolysis reactions which need water obviously, it must be pointed out that water is also necessary for all types of acyl transfers including interesterification even if water is not visible.

The role of the aqueous phase

Water must be present then for two reasons:

- first, enough water must be present in the proteic network to ensure the enzyme its active spatial structure; complete desiccation leads to an inactive structure whose catalytic activity can be restored in general by the addition of water;

- second, a necessary and sufficient amount of water must be associated to the enzyme to reach the optimal thermodynamic water activity "a_w" which allows the enzyme to catalyze acyl transfers excluding hydrolyses where "a_w" is always equal to one.

We should emphasize on the crucial notion of optimal thermodynamic water activity "a_w" in enzymatic syntheses.

Besides the sorption curve of the biocatalyst, the slide is presenting the effect of "a_w" variation upon the activity of the lipase of *Mucor miehei* in a reference reaction of interesterification between pure methylstearate and coconut oil triglycerides *(figure 3)*.

The effect of "a_w" was studied by the determination of the quantity of lauric acid incorporated in the methyl stearate after half an hour of reaction by Muderhwa [2].

The bellshaped curve describes the incorporation of lauric chain in methyl stearate; it shows clearly that, when a_w is near zero, acyl exchanges are becoming nil and beyond the optimal a_w value, competition is arising between hydrolysis and interesterification which finally gives way to the sole hydrolysis when a_w is approaching the value one:

- it is noteworthy to point out the optimum a_w is always located in correspondance with the linear section AB of the sorption curve which corresponds to water molecules ordered in one or several layers involving Van der Waals and hydrophobic bonds with the proteic network;

- in contrast the zone OA ($a_w < 0.3$) corresponds to the sole absorption of water by H bonds on highly polar sites;

- the zone BD ($a_w > 0.6$) corresponds to the apparition of a new aqueous phase absorbed through capillary and osmotic forces. The hachured part is proportional to the quantity of water which wtate is near the state of free water.

Because of the necessity of an aqueous phase in an enzymatic preparation (free enzyme or supported enzyme) one must wonder whether this aqueous phase has to be buffered at a given pH and added up with a surfactant.

As a matter of fact all those parameters must be taken into account for reactions in non aqueous media, because even though the aqueous phase is not visible, it must be present in the enzymatic catalyst as a confined aqueous phase which plays an essential role as described previously and consequently must be buffered at the optimum pH and adjusted at the optimum interfacial tension water/lipid.

Now it is much easier to understand that the aqueous environment of the catalyst must be unchanged whatever

the catalyst or the process may be: solvent, support, the type of reactor (biphasic systems - membrane reactors), the type of reaction which can release water in the medium. The confined aqueous phase of the catalyst must keep then its initial optimum a_w, its optimum pH and its optimum interfacial tension (water/lipid) and of course, all this, for a given reaction in given conditions (temperature and pressure for the main points).

Those considerations are meeting the concept of enzyme memory launched by Yang in 1993 [3].

He showed that the optimum pH of subtilisin in water must be the same inside the confined aqueous phase inside enzymatic particles when working in an organic media; it is likely the same for lipases and other enzymes.

Now we can assess the difficulty to keep constant such properties owing to the fact that free or supported lipases powders are in constant equilibrium with its environment and so that the water content is changing continuously; the rate of water content is also widely dependant on the nature of supports or additives; it is easy to imagine that a preparation of lipase bound to an anionic resin will absorb more water from humid room atmosphere than the preparation of the same lipase bound on to polypropylene beads.

In other words, one must be careful to check water content and a_w before carrying out a reaction with a powdered enzymatic preparation; for this it is recommended to chart the sorption curve of the catalyst. Regarding the pH value of the confined aqueous phase, it is easy to understand that the pH will be dependant on the pH of the mother solution or suspension and should be calculated from the water amount adjusted for a given a_w for a given reaction.

Quality and quantity of interface

Regarding now the interfacial tension, we have to emphasize here also, about some essential points. Because the reaction medium is heterogeneous, the addition of an amphiphilic compound can modify the area of the interface water/lipid (quantitative aspect related to a physical effect) and can also modify the quality of the interface (molecular effect); for example if the substrate cannot reach the interface because occupied by other molecules the reaction will never occur.

The quality and the quantity of the interface is one of the primordial aspects for lipolytic enzymes.

In this respect we are reporting an experiment which was carried out in our laboratory by Pina [4] in the course of 92 dealing with interfacial quality and quantity.

Conditions are shown on the *figure 4*.

Hydrolysis of sunflower oil was studied in a biphasic system in the presence of a surfactant.

Ten BRIJ were tested covering the HLB scale from 4.9 to 18.8. We have chosen a non ionic type amphiphile to avoid ionic interactions with the protein.

The diagram is displaying coarsely a kind of parable showing a maximum hydrolysis either at the lowest HLB value 4.9 (BRIJ 72) or at the highest HLB value 18.8 (BRIJ 700).

This result could be interpreted by the fact that the area of the interface is a crucial parameter as already said above; however we have to admit that the emulsion water in oil obtained for the HLB 4.9 displays about the same area as the emulsion oil in water for the HLB 18.8.

In other words whether the enzyme is in the discontinuous or in the continuous phase, and provided the area of the interface is sufficiently high, the hydrolysis is occurring with a good yield.

Selectivity of lipases

Finally, and before treating of customizing processes of lipases, we will say a few words about the selectivity which can be modified when the environment of lipase is changed.

For example, the enantioselectivity can be changed with the nature of the solvant as reported in 93 by Parida and Dordick [5] who studied esterification of alpha hydroxy acids.

Enantioselectivity can be modified also by the substrate. Verger showed in 93 that *Candida antartica* B lipase is *sn*3 for the hydrolysis of trioctanoin (~90% enantiomeric excess) and *sn*1 for triolein (40% enantiomeric excess) [6].

This very important observation suggests that stereoselectivity could be dependant also on the relative position of fatty acid on glycerol mainly when fatty acids are markedly different.

As a mater of fact if we consider three fatty acids a, b and c, six triglycerides respond to the formula abc.

If we come back to the observation of Verger about the difference between triolein and trioctanoin, and if octanoic acid (caprylic acid) is placed on the *sn*1 position and oleic acid on *sn*3 position just to bother a bit the lipase B of *Candida antartica*, it should be very interesting to see how the lipase would manage depend-ing on whether X is saturated from the shorter chains to the longer ones or unsaturated. One could likely detect whether the relative position of fatty acids has an effect or not and whether the fatty acid of the *sn*2 position has an anchimeric effect on the stereoselectivity of the enzyme. Unfortunately nobody knows, because probably chiral synthesis is still considered as difficult to day in spite of its improvment by Villeneuve [7]. In addition Villeneuve was able to propose a technique to check without any ambiguity, the specificity of lipase through the study of hydrolysis products of a given chiral triglyceride and of its racemic [8].

The *figure 5* is summarizing five couples of pathways defining each a given selectivity.

If the hydrolysis follows the pathway 1 for the chiral TAG, it is still impossible to determine whether the lipase is *sn*3 stereospecific or C typoselective; the undetermination is removed through the hydrolysis study of the racemic. If it follows the pathway 3, the lipase is *sn*3 stereoselective.

In contrast pathways 1 and 4 display a C typoselectivity.

Similarly and following the same reasoning, pathways 2 and 3 shows the lipase is *sn*-1,3 regioselective, 3 and 5 correspond to a *sn*1 stereoselectivity and at least 5 and 6 to an A typoselectivity.

Physico-chemical modifications

Now we are coming to physico-chemical modifications, described on the general scheme given on *figure 6*.

Adsorption

Physical modifications are among the first immobilisations done more than 30 years ago.

Immobilization can be done on inorganic or organic supports. Binding of enzyme is ensured by weak energy bonds namely Van der Waals, hydrophobic interactions or hydrogen and ionic bonds. Mineral supports are the most widely used, and among them silica and glass beads like diatomeous earth, celite, clays and alumina [9-14].

Organic materials such as ion-exchange resins or (bio)polymers are also very widely used.

Immobilisation on Amberlite, Duolite [15, 16], Dowex, Sephadex [17] or chemically modified cellulose are

among the more popular in research world.

Polyethylene, polypropylene and polyurethane and their derivatives are also very used [18-20].

Supports are available as powders, fibers or membranes. The general procedure to immobilise lipases is simple; the enzyme is first dissolved in the desired buffered solution and then the powder is dispersed in the solution under stirring at a given temperature and for a certain time; then the support is dried.

Hydrophobic supports are generally preferred since immobilisation yields are much better. Microporous hydrophobic supports are among the best ones and allow to obtain very good catalytic performance.

For membranes and fibers the buffered solution is passed through and then dried.

Several parameters are governing the success and the efficiency of the immobilisation:

- the size of the protein to be adsorbed;

- the nature of the surface of the support;

- the specific area of the support (porosity, pore size).

Of course the enzyme is adsorbed on the surface and inside the pore as well.

Although the maximum adsorption occurs around the isoelectric pH, one should accept to adsorb less enzyme at a different pH which is necessary for a given reaction.

The final catalyst should have good mechanical properties, a good activity stable for a long time. Desorption should not occur, however there is no rules to predict the efficiency of the adsorption.

Technical interest in using immobilized lipases are summarized in a few words:

- recycling (decreasing costs);

- easy separations after reaction;

- better thermic stability of immobilized lipase.

For lipases immobilisation we have to emphasize on the fact that, since hydrophilic supports compete with the enzyme for the adsorption of water, hydrophobic materials are better supports; for example we have to mention polypropylene which has been described as one of the best provided its conditioning is respected.

For example Bosley [21] showed that it is necessary to treat the PP with a wetting agent and loaded a mixture of ovalbumine and lipase of *Mucor miehei* otherwise the final enzymatic activity is poor.

He explains that, without ovalbumin, the lipase attempts to maximize its contact with the excess surface area available and suffers therefore important conformational changes leading to a loss of activity.

Ovalbumin, the egg protein, blocks the unused adsorption sites and then the adsorbed lipase keeps its active structure.

We would like to mention a natural supported lipase occuring in *Carica papaya* latex [22], namely the crude dried and ground latex which exhibits a powerful lipase activity naturally bonded to a polysaccharide besides its well known proteolytic activity namely papain.

Its lipasic activity was studied and this very low cost material was used in the synthesis of structured

triglycerides by Pina and Villeneuve [23], and also by Mukherjee [24] and Foglia [25].

Speaking of entrapment and microencapsulation, the problem of those systems, is that one needs a great deal of technology, skill and knowledge, and very often a loss of activity or the modification of lipases selectivities are observed.

Moreover the mass transfer through the membranes is somewhat problematic for lipids.

Chemical modifications

A lot of work has been done also in this area, so we will confine our report to some examples only.

Lipases can be bound to water insoluble material or to a polymer matrix through chemical reactions between functional groups of the enzyme and reactive group of an activated material.

Covalent attachment of polyethylene glycol (PEG) to aminogroups of lipase allows to produce an organic soluble lipase.

Inada *et al.* [26, 27] and Baillargeon and Sonnet [28, 29] prepared PEG modified enzymes.

They studied lipoprotein lipase and *Candida rugosa* lipase which were able to solubilize in organic media after modification with activated PEG (APEG).

The amphiphilic nature of APEG makes it possible to modify enzymes in aqueous solutions. The principle is described in *figure 7*.

Inada studied the esterification of lauric acid and lauric alcohol with PEG modified lipase of *Pseudomonas fluorescens* which showed to be a good catalyst for this esterification in chlorinated solvents and benzene where this catalyst is completely soluble.

The same laboratory prepared also magnetic lipase [30] by reacting ferrous (Fe^{+2}) and ferric (Fe^{+3}) ions with PEG modified lipase from *Pseudomonas fragi*. They obtained a tightly bounded PEG lipase to magnetite (chelation).

After reaction the catalyst is of course very easily recovered by a magnetic separation.

This improvement allows to carry out in good conditions acyltransfers reactions in organic media and to separate easily the magnetic lipase at the end; this is of course impossible when the enzyme is under its PEG derivative only.

Fatty acids modified lipases were also prepared by Murakami by reacting aminogroups using the water soluble reagent dimethylsulfodium fatty esters under mild conditions without damaging the enzyme *(figure 8)*. Here is the principle of the lipophilisation of the lipase of *Phycomyces nites* [31].

The modified lipase allows very easy transesterification in hexane, of triolein and different free fatty acids; its activity is 40 times higher than the free lipase.

All the above methods, apart the magnetic technique, are suffering of the difficulty to recover the catalyst at the end of the reaction, so that this very interesting methods are still limited to laboratory work. Different searchers attempted to overcome this major drawback by fixing the enzyme on to a polymer matrix by covalent bonds.

This technique is however not so common as the physical adsorption, it presents the great advantage to avoid the release of free lipase in the reaction medium as often observed for supported lipase linked by adsorption.

The principle is basically the same, and consists of the attachment of the protein through the free amino or carboxyle groups.

Prior to the attachment of the enzyme, supporting materials have to be activated by a chemical. One of the more common chemical is glutaraldehyde which easily polymerizes; the residual aldehyde functions can then react with aminogroups of the enzymes.

Very often a great loss of activity is observed.

However when pancreatic lipase was attached on a partially hydrolysed synthetic polyamide treated by glutaraldehyde [32]. Surprisingly the activity of the final material reached 150% of the initial pancreatic lipase.

Much other techniques could be listed; we will summarize this technique in pointing out that the final activity can vary from almost 0 to 150% depending on the aminogroups which have reacted; of course it is easy to understand that if the active structure of the enzyme is to much far away from the optimum conformation, the final activity will be very poor.

In spite of the advantage of the covalent bonds, the use of sophisticated chemical is still to day a barrier for an industrial development and here also applications are limited to research.

Interestingly, Ito *et al.* customized an enzyme covalently attached on a polymer with a solubility which is changing according to whether the organic medium is irradiated in the visible or UV spectra [33].

The photosensitive polymer is a copolymer of methyl methacrylate, methacrylic acid and a spiropyranyl ethyl methacrylate *(figure 9)*.

Subtilisin modified according to this process is perfectly soluble in toluene and catalyzes very efficiently transesterification of N-acetyl-L phenylalamine ethyl ester with 1 propanol in toluene as solvent.

After UV irradiation the enzyme precipitates and is separated by centrifugation. The solubility is restored after irradiation in the visible spectrum.

Precipitation is performed within 5 minutes and solubilisation within 30 minutes.

The activity of subtilisin is 100 times higher than that of native enzyme.

Process mediated biocatalysts

This type of process can be also a very interesting way to run an enzymatic reaction under controlled conditions allowing to have an influence on the selectivity of a given synthesis or to enhance yield or to work in a continuous operating reactor, etc.

We will give here a few examples of processes which impose a task to the catalyst.

Two phases reactor

We are reporting here a work done in our lab in the years 80 [34-36] about the synthesis of partial glycerides in a biphasic system.

Fatty acids are dissolved in an organic solvent and glycerol in the aqueous phase with the enzyme without any additive. It has been shown that chlorinated solvents (methylene chloride and chloroform) provide a good selectivity for the production of monoglycerides while apolar solvents like heptane and hexane offer a good selectivity for 1-3 diglycerides *(table 1)*.

The authors explain this selectivity by the fact that the reaction occurs at the interface solvent/water and because monoglycerides are first produced *(figure 10)*: in the case of methylene chloride, monoglycerides very soluble in this solvent are leaving very quickly the interface to the bulk of the solvent, on the contrary monoglycerides, poorly soluble in heptane, are staying at the interface and then are acylated again to form 1-3 diglycerides which are very soluble, and can leave now very quickly the interface to the bulk of the solvent.

This system was tested in a continuous operating pulsed column with a company, and results were very good.

Membrane technology

Petrus Carperus and Derksen (ATO-DLO) wrote a very interesting paper on the application of membrane techniques in oleochemistry [37].

Membrane technology can be coupled easily with other techniques and allow then its hybridation with enzyme technology and pervaporation allowing the continuous withdrawal of a volatile product of a given reaction: water for esterification, ethanol for transesterification, etc. *(figure 11)*.

Pervaporation seems well adapted since membrane fouling is seldom encountered in the hollow fiber pervaporation membranes which offer moreover a high packing specific area (up to 30,000 m^2/m^3) and allow the immobilization of large quantities of enzyme.

Through this system, the esterification of a fatty acid with an alcohol can be run with a constant water content in the microenvironment of the lipase, while produced water is removed continuously. Under this process quantitative yields can be obtained.

A microfiltration system is studied actually by Nakajima *et al.* [38]. Oleyl alcohol and oleic acid were esterified with success however the yield is limited to 70%. The lipase used is the lipase from a *Pseudomonas* modified with a non ionic surfactant (alkyl PEG) giving a lipase surfactant complex.

With a similar device fitted with a polypropylene membrane, materializing the interface lipid/aqueous glycerol, Yamane *et al.* synthesized triglycerides according to this process: a conversion of nearly 90% was reached [39].

Lipase mediated oxidation

A few years ago Björkling [40] showed that lipases are able to transfer an acyl group on hydrogen peroxide provided these last are active oxygen resistant.

He showed *Candida antartica* lipase is particularly resistant and able to synthesize peracids. The best result is obtained with lauric acid. If an olefin is present in the reaction medium, the peracid reacts readily on the double bond to form an epoxide and the initial lauric acid is regenerated *(figure 12)*.

The reaction is working very well in apolar solvents like toluene or hexane. Reactions are done at room temperature and only a low concentration of lauric acid and hydrogen peroxide is necessary since lauric acid is regenerated and the level of H_2O_2 concentration automatically regulated.

The yield of epoxidation is almost quantitative for tetramethylethylene.

CONCLUSION

We can say a colossal work has been done to know in depth the lipases structures, to understand their catalytic mechanism and to try to allow the best contact between the catalytic centers and substrates.

Twenty years ago only, Industry was still very sceptic about applications for commodities and it was thought

these techniques would be applied only in pharmaceutical industry.

To day the balance sheet of half a century of hard work, shows that biocatalysis can assist advantageously very common reactions like interesterification for margarine composition or esterification for oleochemicals. These practises which are on the verge to become very common.

In addition we do think that plant lipases will emerge also in the next ten years (papain lipase, castor bean lipase, *Euphorbia characias* lipase, etc.) because their production can be done at low costs from renewable materials eventually through genetically modified plants, and also because some of them are naturally bonded on natural supports like the papain lipase is bonded on a polysaccharide.

However a lot of work is still necessary to go ahead.

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Summary

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Illustrations

Nominated Kaufman Memorial Lecturer at the occasion of the 23rd International Society for Fat research, in Brighton, Jean Graille manages the Lipotechny laboratory of the CIRAD, the French institute for agricultural research for development of developing countries. Author of more than 150 scientific papers, member of several public and private scientific advisory board, Jean Graille is also member of the editorial staff of OCL.

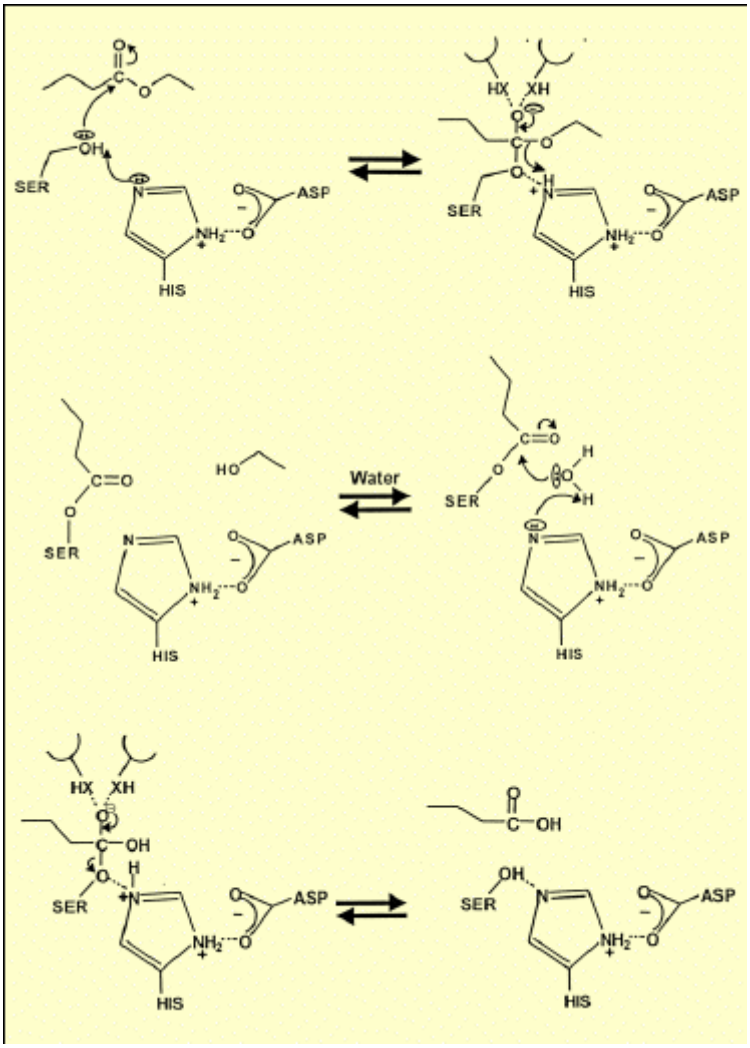


Figure 1. *Triglycerides hydrolysis mechanisms according to Verger [1].*

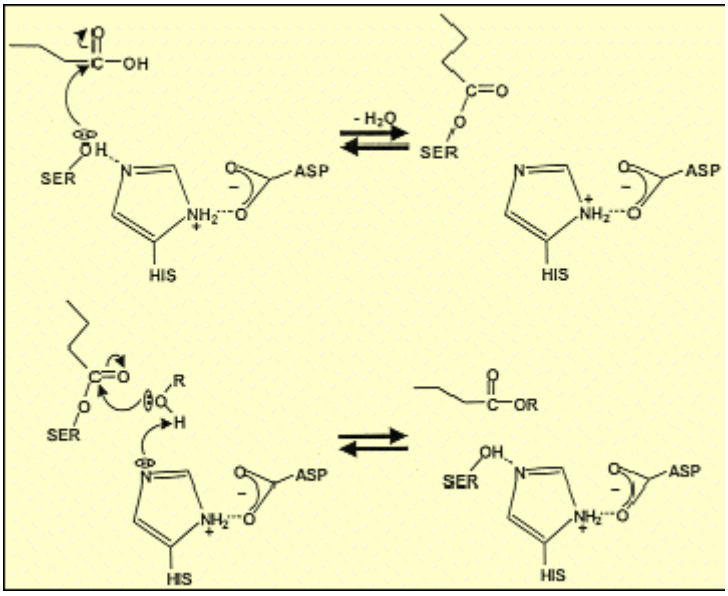


Figure 2. *Esterification mechanism.*

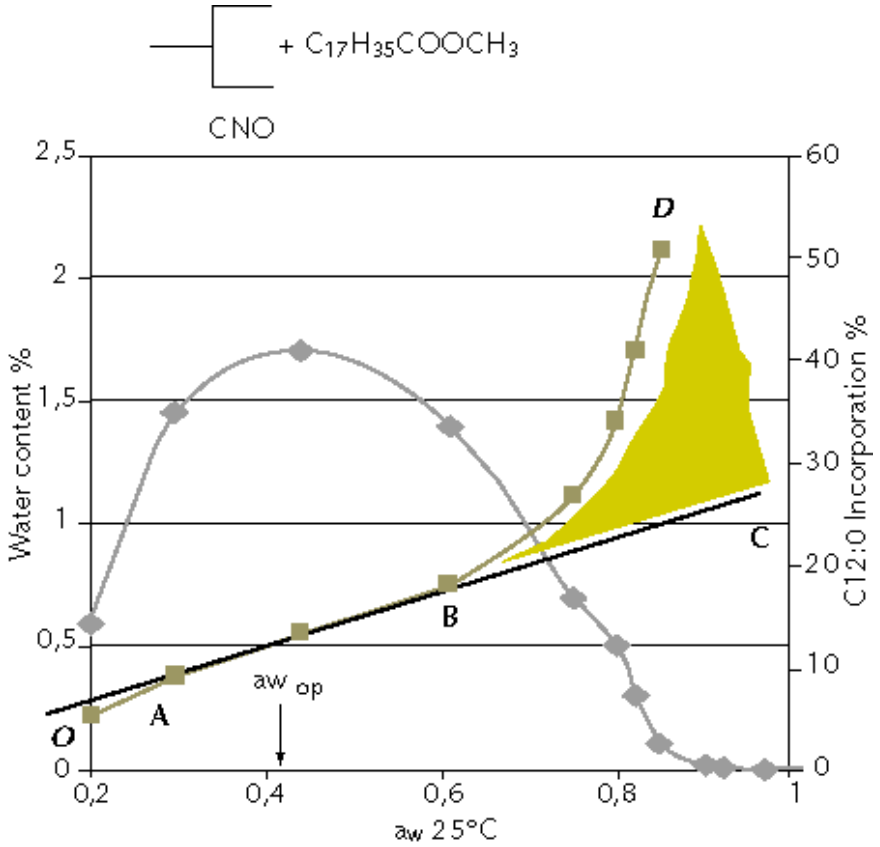
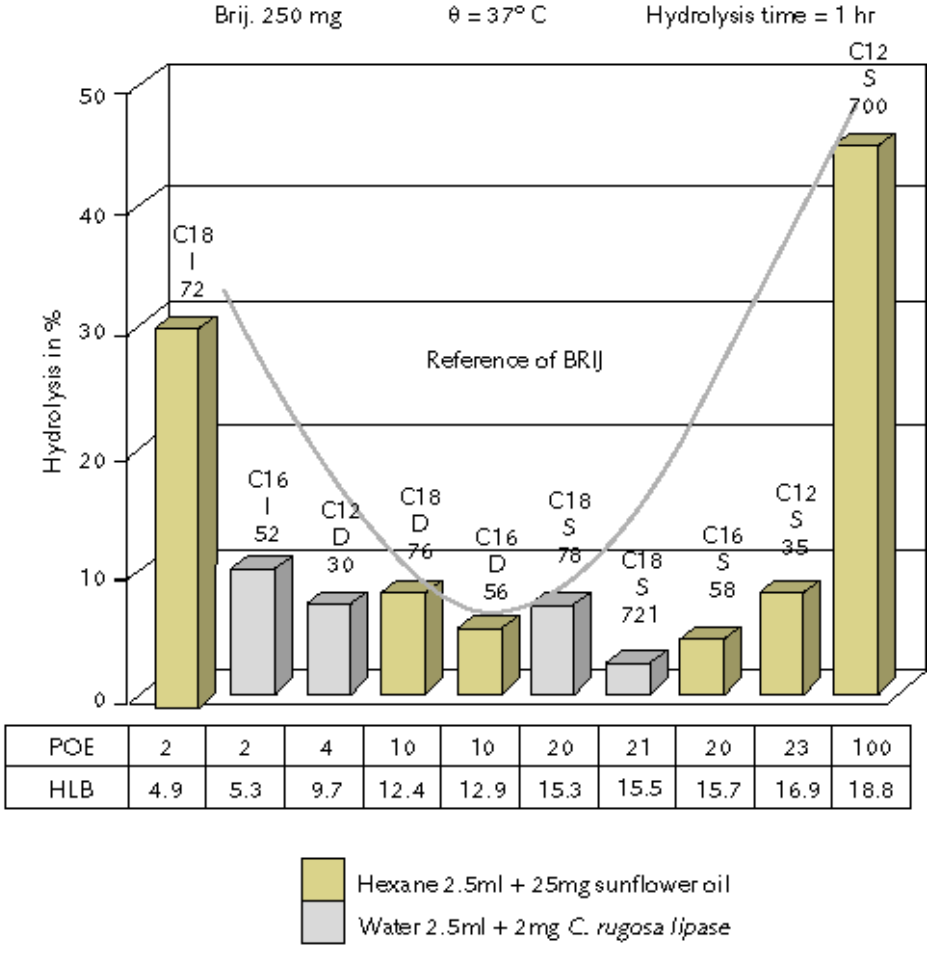


Figure 3. *Role of thermodynamic water activity on the optimal capacity of synthesis for a given lipase according to Muderhwa* et al. *[2].*



POE	2	2	4	10	10	20	21	20	23	100
HLB	4.9	5.3	9.7	12.4	12.9	15.3	15.5	15.7	16.9	18.8

Hexane 2.5ml + 25mg sunflower oil
Water 2.5ml + 2mg *C. rugosa lipase*

Figure 4. *Influence of the interface quality and quantity on the lipolysis.*

POE = polyoxethylen expressed in ethoxyl numbers, HLB = hydrophilic lipophilic balance,

I = insoluble, D = dispersable, S = soluble, C_x indications are showing ethoxylated alkyl chains.

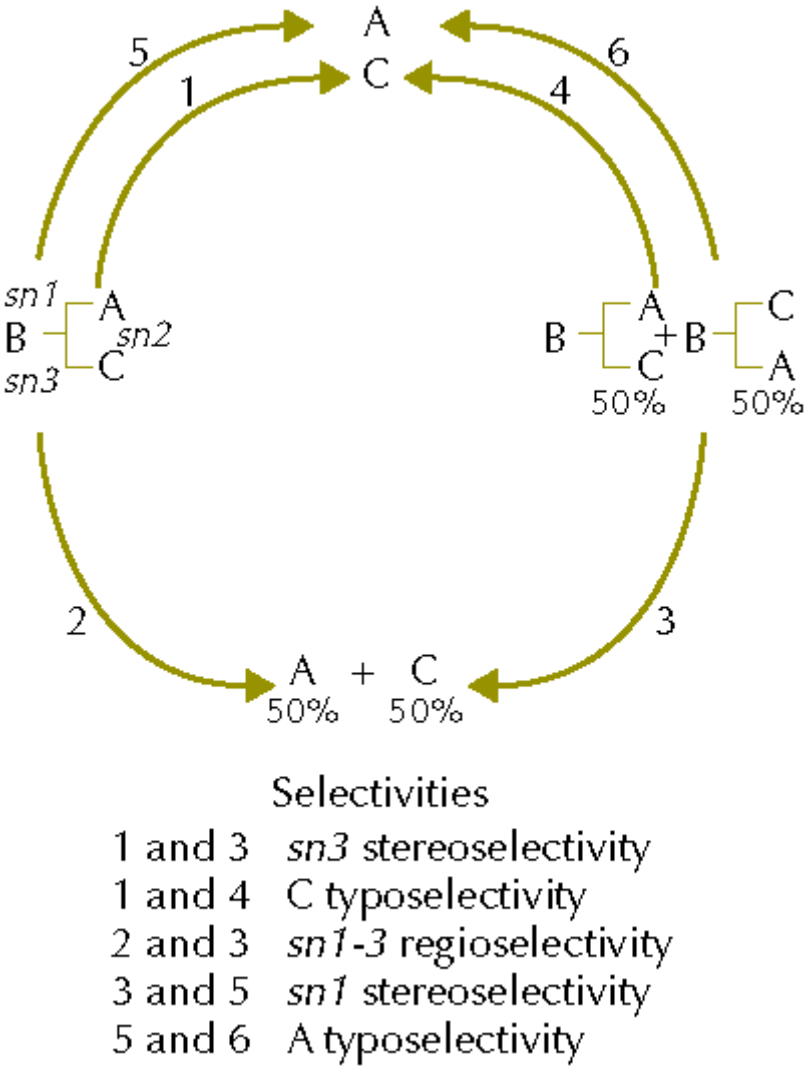


Figure 5. *Selectivity rules according to Villeneuve* et al. *[8].*

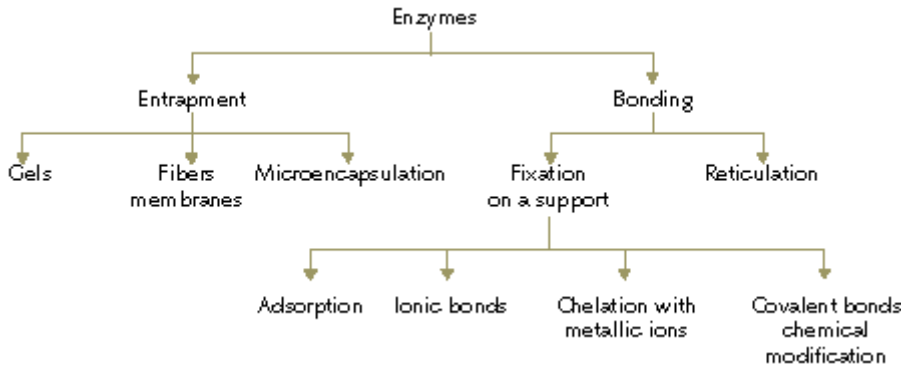


Figure 6. *Classification of the different pathways of immobilization.*

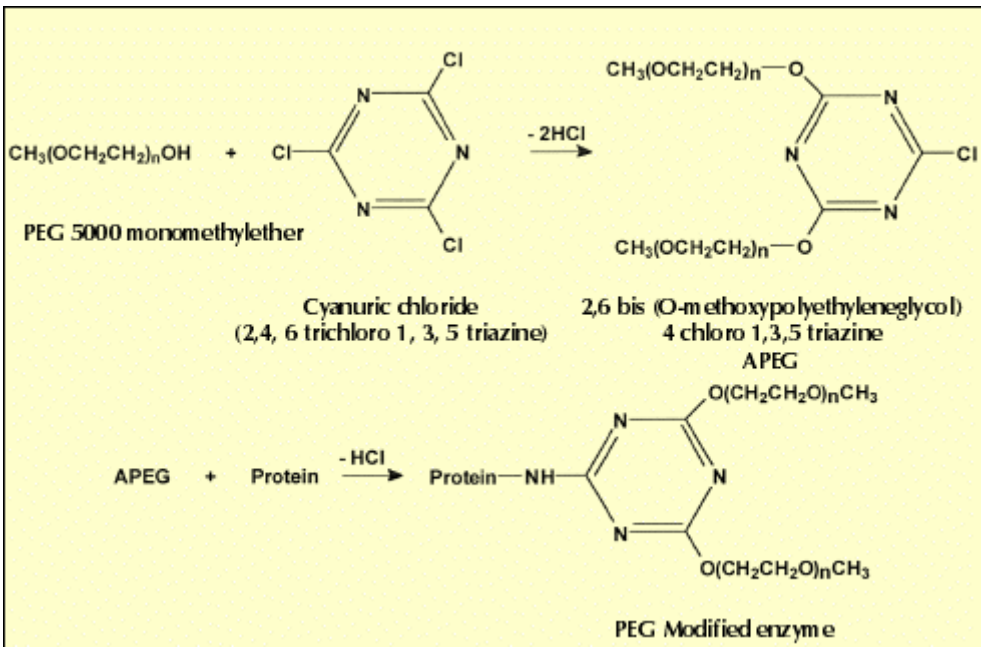


Figure 7. *Modification of enzyme by polyethylene glycol according to Inada* et al. *[29].*

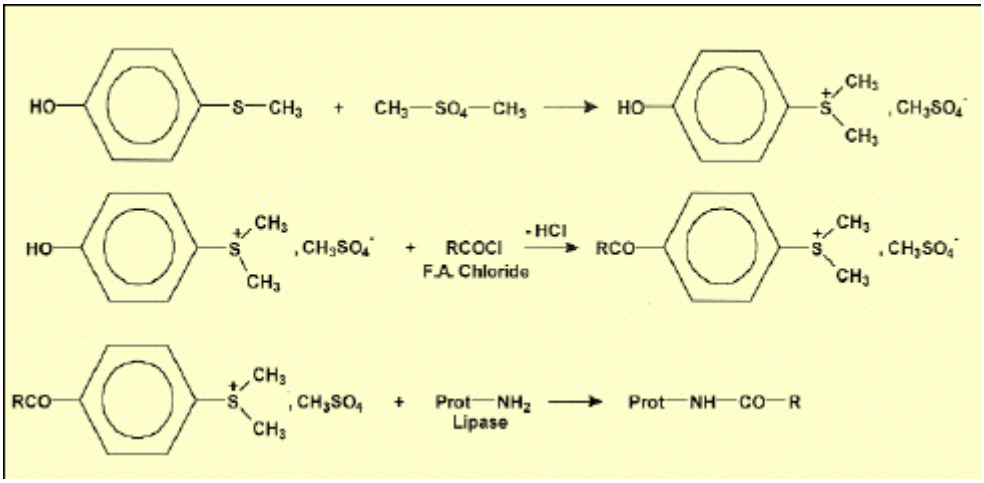


Figure 8. *Modification of enzyme by activated fatty acid esters according to Murakami* et al. *[31].*

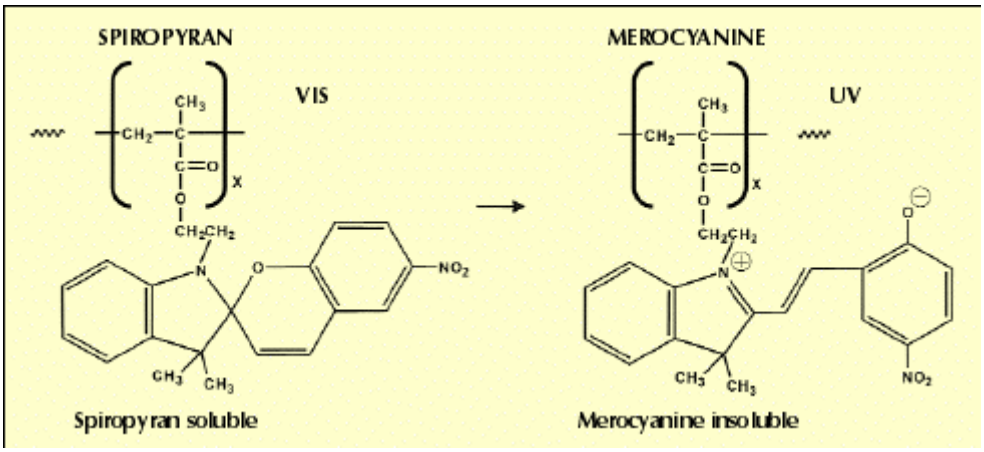


Figure 9. *Immobilization of enzyme on photosensitive polymer with changing solubility according to Ito* et al. *[33].*

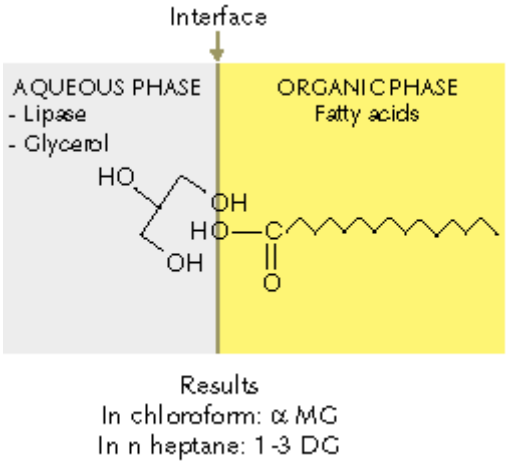


Figure 10. *Esterification principle in a biphasic system.*

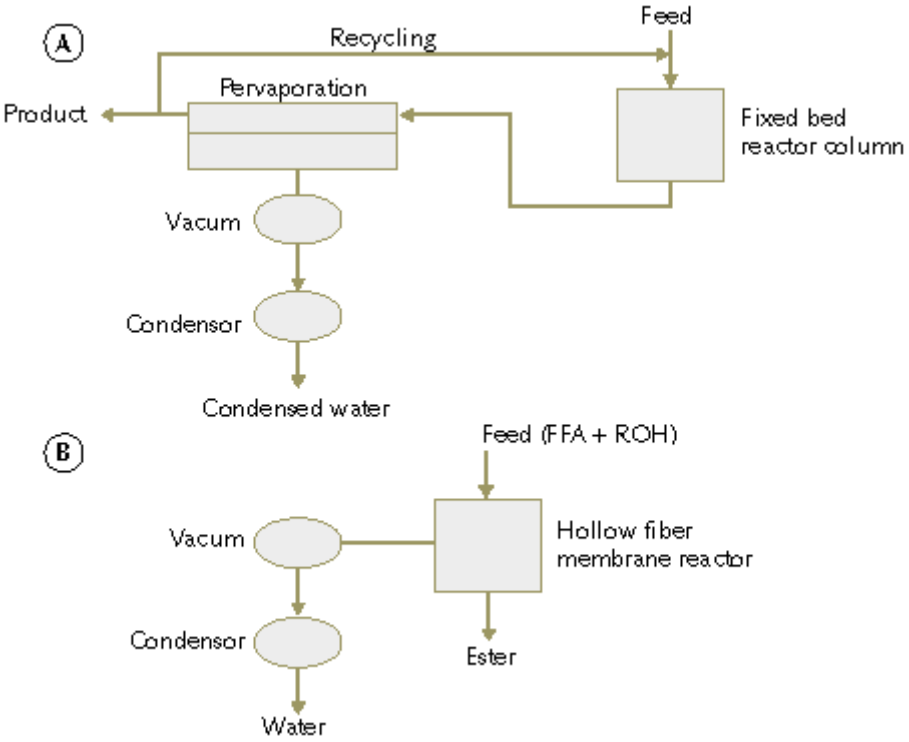


Figure 11. *Hybridation with enzyme technology and pervaporation system.*

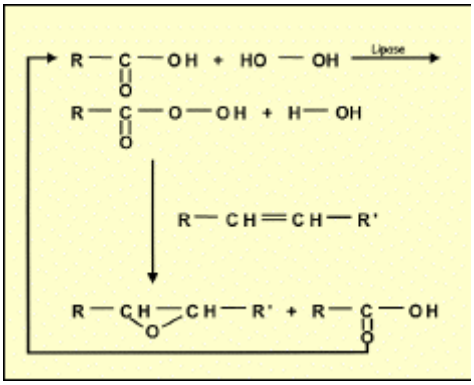


Figure 12. *Olefin mediated oxidation by lipases according to Björkling [40].*

Table 1. *Yields of mono and diglycerids obtained through a biphasic system.*

Conditions	Synthesis of	
	α MG	1-3 DG
Organic phase 150ml	CH_2Cl_2	n Heptane
Aqueous phase 150g	75/25	95/5
(Glycerol/H_2O)	w/w	w/w
R. oryzae lipase 1.5g	120,000 Units	120,000 Units
Temperature (°C)	30	38
Time (hours)	8	4
Yield %	16	27.5
Purity %	100	85