FINE CHEMICALS BY BIOTRANSFORMATION USING LIPASES

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This review paper focuses on the application of lipases for the production of fine chemicals. Lipase characteristics and the various lipase catalyzed reactions involved in the process, such as, hydrolysis, interesterifications and ester synthesis are discussed. An attempt is made to point out the current state-of-the-art, both regarding research in this field, as well as scale-up and commercialization of processes in the fine chemicals industry. Special attention is given to the production of flavors from terpene alcohol which is the current work under development in our research institution.

Keywords: lipases; biotransformations; terpene alcohols.

INTRODUCTION

The term biotransformation can be applied to a specific modification or interconversion of chemical structures performed by enzymes contained in the cells or by isolated enzyme. Biotransformation differs from fermentation in which the substrate is converted to a desirable product through a complex cell metabolic pathway[1,2].

Microbial, plant and animal cells can supply the enzymes for transformations. However, when the permeability of the substrate through the cell membrane is insufficient or when undesired side reactions take place, it is necessary to conduct the biotransformation with cell free enzymes or even with purified enzymes[1].

Enzymes possess several properties that make their use very attractive as a catalyst for biotransformation. They are highly active, versatile and carry out a variety of transformations under mild conditions in a stereo-selective manner. They are generally energy efficient and easy to control[3]. Besides these, recent developments in enzymology, namely, protein engineering and enzymatic reactions in non aqueous media have significantly broadened the scope of potential applications of enzymes as an industrial catalyst[4].

More than 2000 enzymes have been catalogued. Each of them accepts a certain substrate and catalyses a particular reaction which can be classified according to the International Union of Biochemistry[1] into six main groups, as showing in table 1. However their application in organic chemistry is still restricted to a few groups, namely groups that do not require specific cofactor, such as, the hydrolysing enzymes. Among this, the use of lipases as biocatalyst has been subject of increasing interest.

Currently, lipases represent no more than 3% of all enzymes used in industry. However, this percentage is expected to increase as processes are being reevaluated in the light of their feasibility of working in organic solvents[5].

The application of many types of enzymes[6-9] and in particular of lipases[10-15] in organic synthesis has been already reviewed by several researchers. In addition, a large number of papers dealing with several aspects of the use of these biocatalyst in non-aqueous media are also available[16-19]. Figure 1 shows the dynamics of an increase in the numbers of reviews devoted to the behavior and properties of biocatalyst in organic media[19].

Table 1. Classification of the enzymes according to the International Union of Biochemistry.

Group of Enzyme	Reaction type
Oxidoreductases	Catalyze oxidation reductions reactions involving oxygenation, such as, CH→C-OH, or overall addition of hydrogen atom equivalents as for CH(OH)→C=O and CH→C=C.
Transferases	Mediate the transfer of groups such as acyl, sugar, phosphoryl and aldehyde or ketone moieties from one molecule to another.
Hydrolases	Promote hydrolysis of esters, amines, lactones, ethers, peptides and other C-N containing functions. Hydration of C=C bonds and epoxides.
Lyases	Catalyze additions, usually of HX, to double bonds such as C=C, C=N and C=O, and the reverse reactions.
Isomerases	Migration of double bonds or oxygen functions, racemization and rearrange ments.
Ligases	Mediate the formation of C-O, C-S, C-N, C-C, and phosphate ester bonds.

This review will highlight some recent progress made in our understanding of the processes catalyzed by lipases in organic media and identify current applications of this versatile enzyme.

LIPASE CHARACTERISTICS

Sources

Lipases have been found in various tissues of many animals and plants. They can be also produced by fermentation processes using several microorganism species, namely fungi and bacteria. From economic and industrial standpoints, microorganisms are preferable to animals and plants as enzyme sources. In fact, various microorganisms were found to be efficient lipase producers and consequently, microbial lipases are commercially available in large quantities for industrial use[20]. Some examples of commercial lipase preparations are presented in table 2.

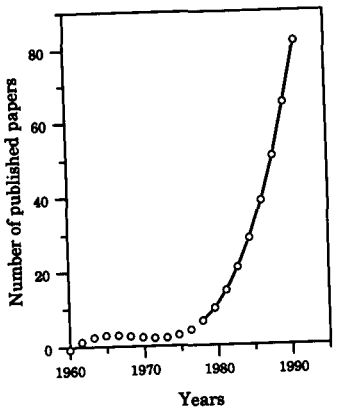

Figure 1. Increase in the number of review papers published during 1960-1990 dealing with the behavior and properties of biocatalyst in organic solvents [Reference 19].

Table 2. Examples of available and reported commercial Lipase preparations.

Sources	Brand name	Company
Aspergillus niger	Lipase AIE	Amano
Rhizopus japonicus	Lipase Saiken	Osaka Saiken
Rhizopus arrhizus	Lipase 80000	Gist Brocades
Rhizopus delemar	-	Sheikagu-Kogyo
Mucor miehi	Lipozyme	Novo Nordisk
Candida cylindracea	Lipase OF 360	Meito Sangyo
Pseudomonas sp.	Lipase LPl	Amano
Choromobacterium viscosum	Lipase T-01	Toyo Jozo

Although, research development is more concentrated on the application of these enzymes, at present, there exist some investigations dealing with lipase production. In this sense, it is important to mention the work being developed at GBF (Braunschweig/ Germany) on the isolation and identification of heat-labile lipases from psychrophilic bacteria originated from the German polar vessel "Meteor"[21].

Classification

Lipases belong to the serine hydrolase group of enzymes (EC.3.1.1.3). Triglycerides are their natural substrates and their mode of action is related to their interfacial properties in biphasic systems[20]. Lipases may display a totally different substrate spectrum in organic solvent or even more important catalyze reactions that are hardly possible in water, such as transesterifications and esterification[1]. The reactions mediated by lipases are showed in figure 2. High regioselectivity and stereoselectivity have been observed with lipase catalyzing reactions in organic media[1].

Lipase Specificity

The specificity of lipases is often crucial to their application for analytical or industrial purposes. The enzymes can show specificity with either the fatty acyl or alcohol parts of their substrates[20]. Usually, they can be classified into three groups according to the regiospecificity exhibited with acylglycerols substrates. They can be non-regiospecific or sn-1, 3-specific toward triglycerides and they can possess specificity toward particular types of fatty acids[22,23].

Lipases belonging to the first group show no specificity regarding the position of the ester bond in the glycerol molecule or the nature of the fatty acid. They catalyze the complete

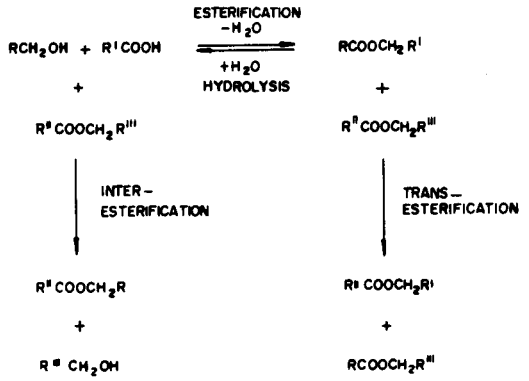

Figure 2. Application of lipases for completing various reactions.

breakdown of triacylglycerols to fatty acids and glycerol, but diacylglycerols and monoacylglycerols are intermediates in the reaction (Figure 3-a).

Enzymes in this group include the lipases produced by *Candida cylindracea, Propionibacterium acnes, Staphylococcus aureus.*

The second group of lipases releases fatty acids regiospecifically from the outer 1 and 3 positions of acylglycerols (Figure 3-b). With these lipases, triacylglycerols are hydrolysed to give free fatty acids, 1,2(2,3)-diacylglycerols and 2-monoacylglycerols. The rate of hydrolysis of triacylglycerols is normally faster than that of diacylglycerols, and consequently substantial quantities of both diacylglycerols and monoacylglycerols accumulate during the reaction[10]. Since 1,1(2,3)-diacylglycerols and 2-monoacylglycerols are chemically unstable, they isomerize yielding 1,3-diacylglycerols and 1(3)-monoacylglycerols respectively. Prolonged reaction of this fat with a 1,3-regiospecific lipase results in a complete breakdown of some of the triacylglycerols with the formation of glycerol. 1,3-regiospecificity is common among extracellular microbial lipases, and enzymes of this type are produced by *Aspergillus niger, Pseudomonas fluorescens, Humicola lanuginosa, Chromobacterium viscosum* and various *Rhizopus* and *Mucor* species[22].

Most extracellular microbial lipases are not specific regarding the type of fatty acid released from common natural oils and fats. However, the lipase excreted by the mould *Geotrichum candidum* can be considered as exception because it has a marked specificity for the hydrolysis of esters of a particular type of long chain fatty acid. Preferably, the enzyme releases Δ-9 fatty acids from triacylglycerols. Satured fatty acids and unsaturated fatty acids without a double bond in the 9-position are only released from triacylglycerols. Generally cis-Δ9 fatty acid esters are hydrolysed more rapidly than their trans-Δ9 isomers.

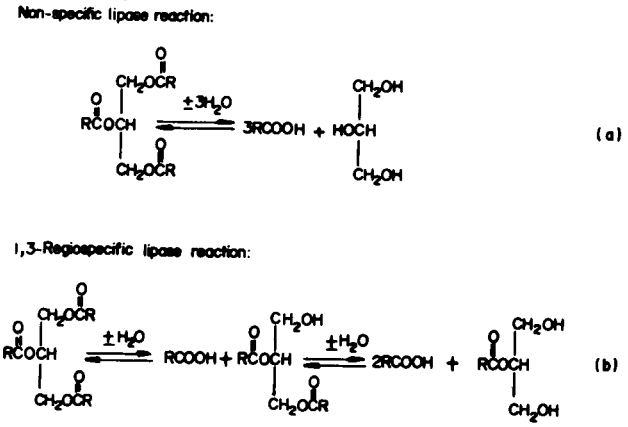

Figure 3. Regiospecificity of lipases

When *G.candidum* is used as an interesterification catalyst only cis-Δ9 fatty acyl groups are exchanged in mixtures of triacylglycerols or triacylglycerols plus fatty acids[22,23].

Stereoselectivity

The ability of enzymes to discriminate between enantiomers in a racemic mixture has been frequently employed for the resolution of racemate, a task which is difficult to achieve by chemical methods[1]. Lipases have been used to perform stereospecific reactions to yield optically pure aliphatic and aromatic esters, alcohols, acids and lactones[24]. These reactions may be important as one isomer of certain compound may have desirable attributes than the other. For example, the (R)-isomer of aspartame has a sweet taste while the (S)-isomer has a bitter taste attribute. The enantiospecificity of several lipases is known, yet cases exist where the enzyme's specificity changes with different ester substrates. This change has been related to the chemical nature of the ester, particularly its chain length[24]. In addition, it is essential to choose appropriate organic solvents both to dissolve the reactants and to shift the equilibrium toward esterification[25]. In figure 4 the stereospecific esterification by using lipase for the resolution of the racemic mixture of (R/ S) menthol is illustrated[26].

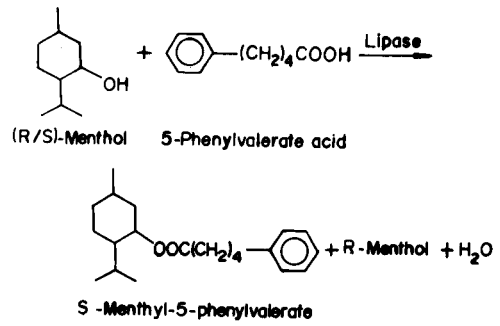

Figure 4. Steoreoselective esterification of (R/S) menthol catalyzed by lipases in water satured isooctane, 100% e.e. [Reference 26].

Properties

Depending upon their sources, lipases may have molecular weights ranging from 20,000 to 200,000 and are active in a broad pH range (4-9) and temperature varying from ambient to 70°C. Generally, they are surface active enzymes and can only be activated by binding to interfaces of long chain fatty acids and water, which serves as their natural substrate in the aqueous phase. This and the ability to act on long chain fatty acid esters are used to differentiate lipase from esterase[20].

Production

Some of the lipase so far studied are inducible, therefore their synthesis is regulated. Their production is suppressed by the presence of simple sugars such as glucose and fructose or glycerol in the culture medium. On the other hand, fatty acids and lipids including triglycerides (olive oil, ground nut oil, etc.) can effectively stimulate lipase production or synthesis. Media are formulated using complex polysaccharides such as starch or bran, triglycerides or fatty acids as carbon and energy source. Nitrogen sources most frequently used come as peptone, meat extract or casein hydrolysate.

The lipase produced using such types of media may not be freely released from the surface of the cell wall of the organism. Their presence on the cell wall can inhibit further production of the enzyme. Therefore, release from the cell wall is affected by incorporating high levels of Mg^{+2} to the growth medium. This approach works for the lipase of *Geotrichum candidum*. Also, addition of lecithin to growth *Rhizobium japonicum* causes accelerated excretion of the enzyme[20].

Lipase Assay

Different substrates can be used for the assay of lipase, but the preferred methods use emulsions of insoluble triglycerides which are liquid at the assay temperature (usually lower than 40°C). This is because of the slow rate of hydrolysis of solid triglycerides and use of soluble esters in lipase assays lead to erroneous conclusions. The activity of the lipase is measured either titrimetrically or colorimetrically[27]. The titration method based on the amount of free fatty acids released and in the quantity of base required to neutralize it. The colorimetric method employs conjugated substrate that upon hydrolysis releases a chromophore such as p-nitrophenol (*p*-NP). The yellow color of *p*-NP produced can be spectrophotometrically determined at 410 nm. The reagent containing the substrate must, however, be used immediately to avoid phase separations[27]. There are also other methods which use fluorometry and radiometry but these are used in more selective investigations[21].

Thin layer chromatography using silic acid plates and extracted products of lipolysis can be used to determine the positional specificity of a lipase[28]. A better approach to the study of positional specificity involves incubation of the lipase with mixed acid glyceride of a known structure. The reaction products are then isolated on TLC and the fatty acid composition determined by GLC[29].

ADVANTAGES OF USING LIPASE IN ORGANIC CHEMISTRY

Lipases are a particular useful category of enzymes in organic synthesis, they accept a broad spectrum of substrates, including lipophilic substances and, in addition, they are relatively stable in aqueous and even in organic media where lipophilic substances can be readily processed[1,20]. For example, porcine pancreatic lipase is stable for several hours in toluene (log P = 2.5) or decanol (log P = 4.0) even at 100°C. Lipases may display remarkable novel properties under anhydrous solvents. Thus, lipases can be used in the reverse mode, i.e. for ester synthesis, interesterifications and resolution of racemic mixture to produce optically active compounds[10-14]. This knowledge has enormous potential for several industrial applications as will be later described in this paper. Apart from, synthesis being favored over hydrolysis, there are several other advantages to employ lipases in organic media[1,3]; increased solubity of non-polar substrates is one and an easy enzyme recovery by filtration is another[10,16]. Product recovery is also easy from low-boiling solvents and the possibility of carrying out a reaction solvent-free is another highly valuable advantage[30,31]. According to several workers[3,10] the optimal moisture content control is the key factor for success with the various process catalyzed by lipases in organic media. It affects variables such as reaction rate, extent of hydrolysis side-reaction and yields of product, selectivity and stability of the enzyme under operation conditions[31-34].

ASPECTS OF LIPASE STABILITY AND ACTIVITY IN ORGANIC SOLVENTS

It is generally accepted that each biocatalyst is surrounded by a thin layer of lightly bound water. The presence of this layer (also called hydration shell) is considered to be a pre-requisite for the retention of enzyme activity in organic media[19]. Therefore, the water content of the system is a very important parameter and can dictate not only the enzyme activity but also its stability[16,22].

When lipase is used as a catalyst, the influence of water content in the system becomes more important due to the reversibility of the lipase activity which also depends on the water content in the mixture. At high water content the hydrolytic reaction prevails whereas at low water content the synthetic reaction is favored. Thus synthesis of ester is associated with many difficulties since there are three potential sources for the presence of water in the system that has to be considered: solvent, enzyme and reaction water resulting from ester production.

Considering the reaction difficulties above mentioned and the notion that an undisturbed hydration shell is a pre-requisite for successful functioning of an enzyme in a non-aqueous environment, the following principles for constructing biocatalystic systems in organic media have been suggested[19].

Solvent Selection

The single most important criterion in selecting a non-aqueous solvent is its compatibility with the maintenance of the catalytic and substrate specific to the enzyme. Therefore, the selected organic solvent should not strongly disturb interactions in the hydration shell of the enzyme that is necessary for maintaining its native conformation[35].

Solvents used to carry out reactions catalyzed by lipases include benzene, toluene, n-hexane, cyclohexane, heptane, octane, iso-octane, nonane, petroleum ether, triacetin, acetone, carbon tetrachloride, chloroform, ethyl acetate among others[36]. The reported enzymatic reaction rates using such solvents are strongly affected by the polarity of the organic media. Such results follow the consensus that high biocatalyst activity is attained in relatively hydrophobic solvents and none or low activity is observed in relatively hydrophilic solvents, as following illustrated[22,37].

HIGH ACTIVITY ↔ HYDROPHOBIC SOLVENTS
LOW ACTIVITY ↔ HYDROPHILIC SOLVENTS

For practical purposes, the most simple method to quantify hydrophobicity is log P, defined as the logarithm of the partition coefficient in a standard octanol-water two-phase system.

$$P = \frac{[solute]octanol}{[solute]water}$$

Log P values are either known or can be calculated from hydrophobic fragmental constants. Besides this, it has been demonstrated that a clear sigmoidal-shaped correlation exists between enzyme activity retention and log P, as shown in figure 5. Activities are low in relatively hydrophilic solvents with log P < 2, is quite variable in solvents having a log P between 2 and 4, and is high in hydrophobic solvents with log P > 4. Based on these results, it was concluded that only about 20% of the commonly used organic solvents are suitable for general use in biosynthesis[37,38].

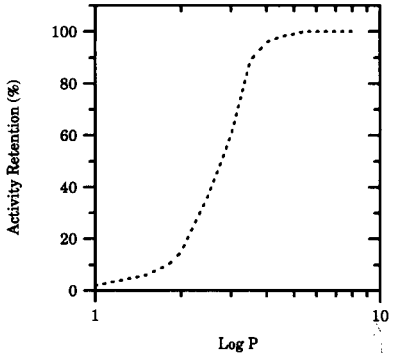

Figure 5. Schematic plot between activity retentions of biocatalyst exposed to organic solvents versus log P [Reference 37].

Enzyme

If a solvent does destroy the hydration shell the conformation of the enzyme should be artificially fixed, so that its conformation remains native (or at least close to native) even in a partially dehydrated state. This can be achieved by using one of the following methodologies.

Spatial Separation

The catalytic properties of an enzyme are retained if the hydrated enzyme and the organic solvent have separate locations in the reaction system, i.e. the direct contact between them is prevented.

Reverse micelles is just one example of a system in which the principle of spatial separation of enzyme and organic solvent is used. Such kind of system deals with the catalysis by enzymes entrapped in hydrated reversed micelles of surfactant (detergents, phospholipid, etc) in organic solvents. The retention of enzymatic function in such microheterogeneous media is not surprising in lipase, since the presence of an interface is an obligatory condition for its functioning. As shown in figure 6, surfactant molecules contain a hydrophilic "head" and a hydrophobic "tail"[39]. When dissolved in apolar organic solvents, surfactants spontaneously aggregates to form reversed micelles, which consist of some tens of molecules oriented in such a way that the heads point inwards and the tails outwards. On addition of small amounts of water, these aggregates swell to form microdroplets of water surrounded by a spherical monolayer of the surfactant. This system is a water-in-oil (w/o) microemulsion and is transparent and thermodynamically stable[19,40,41].

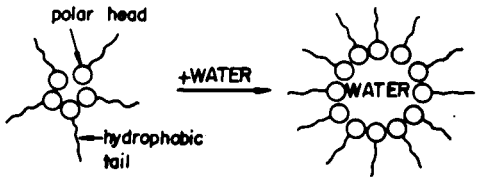

Figure 6. Solubization of water by reversed micelle in organic solvent [Reference 39].

Enzyme Immobilization

Another way to prevent hydration shell disruption is to fix artificially the enzyme configuration, by applying immobilization techniques. Immobilization is believed to stabilize the enzyme because of the chemical and physical interactions between enzyme molecules and supporting materials[38]. Immobilization also helps to keep the dispersion of enzyme in organic solvents homogenous, which is essential for efficient enzymatic reactions. Moreover, immobilized enzymes can be used continuously or repeatedly as catalysts in biorectors[36,38]. Many methods for achieving the immobilization of lipase are available[42-45] and addition information concerning the feasibility of such methods can be found elsewhere[38].

Reaction water resulting from ester production

The water generated through the reaction is considered to be the most important parameter that needs to be controlled. Even under conditions of low water concentration, during the course of the reaction, 1 mole of water is formed for every mole of ester synthesized. In monophasic organic media, the water produced is distributed as follows: it is adsorbed by the solid enzyme phase and it also dissolves in the solvent up to the saturation level; then it forms a separate aqueous phase. The appearance of this phase is dependent on the capacity of

water adsorption by the lipase preparation and on the hydrophobicity of the solvent. As hydrophilic solvents are usually avoided to prevent enzyme inactivation, the formation of this discrete aqueous phase can occur early during the reaction[46]. Removal of water by several methodologies can prevent this phenomenon and help to drive the reaction to its completion with high yields[47-54].

Control and removal water product has been performed by several workers, including strategies such as nitrogen or air sparging[47,48], simple evaporation[48], molecular sieves[47-50], distillation[50] and vacuum stripping[48,51].

An alternative solution has been demonstrated by Halling's research group[52-54] where salt pair hydrates are used to buffer water level during lipase catalyses synthesis. This has been particularly useful to fix and control the water parameter for several reactions, although each system requires carefully study. For example, there are pairs of salts that have poor temperature stability such as Na_2SO_4 and pair of salts which can react with the substrates such as Na_2CO_3. A special guide for the proper use of this methodology has been published[54]. There are, however, some catalyzed synthesis that is not so sensitive to the change of water content in the whole system. This is the case for some immobilized catalysts, specially those with high hydrophilic values[55,56], such as Lipozyme, a commercial available enzyme preparation manufactured by Novo Nordisk[57].

The application of a suitable strategy for water removal during the course of the esterification is still subject of intensive experimental work by several researchers and depends on the model of the system used (enzyme preparation/ alcohol/ fatty acid/ solvent). Comparative performance of some these water removal strategies have been discussed in the case of synthesis of terpene butyrates[58].

INDUSTRIAL APPLICATIONS

Representative and possible applications for lipases are shown in table 3. As can be seen, lipases can be used in food, cosmetic, beverages, chemicals, pharmaceutical, leather, medical, waste water treatment and detergent industries[20,59]. Examples of industrial interest are given as an illustration of the potential application of lipases.

Cocoa butter equivalents

Chocolate contains 30% cocoa butter, and this kind of fat confer on chocolate its required crystallization and melting characteristics. Cocoa butter is very expensive, and the food industry has developed fat mixtures, known as cocoa butter equivalents, which can be used for cocoa butter in chocolate and related products[60]. A typical method (Figure 7) for producing cocoa butter equivalents consisted in mixing palm mid fraction and stearate ester, followed by dehydration and enzymatic reaction. After distillation, the gained triglyceride fraction was solvent fractionated and a low melting point fraction was obtained as cocoa butter-like fat[60,61]. This process has been proven to work at lab-scale since mid-1980s with Novo Nordisk Lipozyme, and is now working in a commercial production by Loders Croklaan of the Unilever Group, in Wormerver, the Netherlands[62].

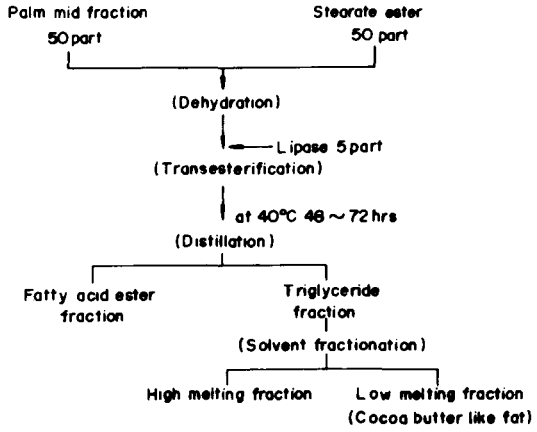

Figure 7. Scheme for the production of cocoa butter like fat by lipase

Interesterification of Fats and Oils

Interesterification is used commercially to alter the physical and functional properties of fats and oils. Industrial process is done by heating oils and lipid mixtures free of fatty acids and peroxides with chemical catalysts (e.g., sodium metal). Interesterification of fats and oils can also be achieved by using lipase,

Table 3. Potential uses of lipases.

Industrial Field	Effect utilized	Product
Food		
Dairy	Hydrolysis of milk fat	Flavoring agent
Bakery	Improvement of flavor/qualities and prolongation of shelf life	Bakery products
Brewing	Improvement of aroma and acceleration of fermentation, by removal of lipids	Alcohol beverages, e.g.; sake,wine
Dressing	Quality improvement of egg by lipid hydrolysis	Mayonnaise, whipping
Meat and fish processing	Development of flavor, and removal of excess fats	Meat and fish products
Chemical		
Oil processing	Transesterifications of natural oils	Oils or fats
Fine chemicals	Synthesis of esters, resolution of racemates	Esters
Detergent	Digestion of oil stains/ spots and lipids	Detergent for household uses
Miscellaneous		
Pharmaceutical	Digestion of oil and fats in foods	Digestant
Medical	Blood triglyceride assay	Diagnostics
Cosmetic	Removal of lipids	Cosmetics in general
Leather	Removal of fats from animal skins	Leather products
Environmental	Decomposition and removal of oily substances	Waste water treatment and others, in combination with other enzyme

and this method has many advantages over the traditional chemical methods, such as: selective and positional specificities, fatty acid specificities, milder reaction systems and tolerance to free fatty acids.

Research on this subject has been carried out by several workers[63-65] using commercial immobilized lipase (Novo Nordisk, 1.3-specific enzyme) for hydrogenation of Canola Oil[63,64] and for incorporation of palmitic acid into babassu fat[65].

Evaluation of biodestructive plastics

A kind of material that can be broken down into innocuous product by microorganism action is classified as biodestructible[66]. Polyethylene mixed with starch, widely used in fast food restaurants, is a typical biodestructive plastic. The main difference between biodestructible and biodegradable plastic is the degree of decomposition that can be achieved by such kind of materials. An incomplete degradation is obtained for biodestructible plastics whereas a total degradation is observed for biodegradable plastic[66].

The conventional technique for the evaluation of the destructibility of a given plastic takes several months. It consists in buried the plastic sample in soil and observing the degree of its decomposition. Due to the variations on the soil conditions through the analysis period, data comparison is normally precarious[67]. A new technique to assess plastic degree of destructibility has been established by the Fermentation Research Institute (Tsukuba/ Japan), using lipase which has the ability to ready degraded polycaprolactone (a kind of aliphatic polyester). Changes on the degree of destructibility are observed when polycaprolactone is mixed with other kinds of plastics and this difference can be used to evaluate the level of destructibility of a given kind of plastic. Such methodology has been successfully applied for various biodestructive plastic determinations. In addition to this, the technique can be also applied for determining kinds of materials that can be mixed with plastics in order to increase its degree of degradability[68].

Production of Monoglycerides

Monoglycerides, which are widely used in food industry as emulsifier, are produced by base-catalyzed glycerolysis of triglycerides at high temperatures. The reaction mixture is then neutralized, and the product is isolated via molecular distillation. An alternative process is based on lipase catalyzed alcoholysis of triglycerides. The enzymatic reaction takes place in 95% ethanol to yield the monoglycerides and the corresponding ethyl esters of fatty acids of C 1,3 positions. The product is isolated by simple filtration or crystallization. This process has been developed from various fats and oils. The reaction yields and product purity are typically high (>90%). Triolein has been used as a model compound obtaining monolein in high yield with minimum amount of glycerol as by-product[69,70].

Enrichment of Omega-3-Fatty Acid

The polyunsaturated fatty acids: omega-3 fatty acids, cicosapentaenoic acid and dosahexaenoic acid in fish oil have been under extensive research work to determine the health benefits associated with them[71]. Using similar methodology as described above, it is possible to achieve enrichment of such somewhat polyunsaturated fatty acids. The starting material, e.g., men handen oil, containing about 25% of omega-3 fatty acids can be subjected to the lipase-catalyzed alcoholysis reaction, followed by separation of esters and cholesterol and fractional crystallization. The product obtained can achieve 60-70% in omega-3-fatty acids[72,73].

Chiral Synthesis

Asymmetric synthesis is one of the major themes in organic chemistry. It has long been known that biological activity of the enantiomers of a drug may differ. Moreover, the undevised enantiomer can have a different type of activity or cause toxic side effects. Nevertheless, the majority of synthetic chiral drugs are marked as racemate as illustrated in table 4 [9,74]. Until recently the synthesis and chemical development of optically pure drugs faced tremendous difficulties and was uneconomical. However, development of new methods of asymmetric synthesis and chiral analytical techniques, as well as mounting pressure from regulatory agencies in the United States, Europe and Japan are rapidly changing the status quo.

Table 4. Synthetic generic drugs with a single chiral center.

Merck Index (enantiomeric indication)	Compound
None (sold as racemate)	Atenolol Chlorthalidone Guaiafenesin Hydroxyzine Ibuprofen Lorazepam Meclizine Metaproterenol Promethazine Propranolol Temazepam Terbutaline
In text but not in name (sold as racemate)	Albuterol Miconazole Warfarin
In name (sold as racemate)	Fenoprofen Metoprolol
In text, sold in chiral pure form	Methyldopa Naproxen Phenylephrine hydrochloride

There are basically two different ways to change a product from a racemic mixture to a single compound. For instance, the manufacturing process could be drastically altered so that only the desired chiral product is produced. The alternative leaves the existing manufacturing process in place and resolves the (R/S) mixtures. In this case the lipase-catalyzed kinetic resolution of racemate offers an easy way to perform synthesis of important biologically active compounds in organic solvents[74,75].

Several chiral building blocks of important drugs such as fluoroprostaglandins, leukotriene, antagonists, adrenergic agents and others have been synthesized using stereoselective hydrolysis in water or stereoselective transesterification in non-aqueous media[76,77].

Paper Manufacturing

Besides the use of cellulases and lignases in the paper manufacturing industry[78], the use of lipases to improve the paper process has recently been explored[13]. Deposition of resins on drying cylinders is, sometimes, a serious problem affecting paper quality and productivity. Treatment of the pulp with lipases lead to a considerable improvement in productivity and sustained less frequent cleaning of the drying cylinders required for the process. Besides the technical advantages of this mild and safe procedure, a shorter storage

of the wood and reduction of the amount of the other chemicals used in the pre-treatment of resins have been achieved[13].

Flavor and Fragrance Production

Most of the desirable flavoring and fragrance compounds are esters. Commercially viable routes for their production should use a generic system with only minor modifications needed to adapt it to the production of the required ester. Lipases have been used to produce esters with different functional properties from low molecular weight flavor and fragrance compounds[79-81] to specialty wax substrates of varying lengths, structural side chains and levels of saturation[82-84].

Alternatively, flavors can be produced on integrated schemes for conversions involving several steps. Such kind of process is under currently investigation by several workers[85,86] and takes advantage of the use of lipase for ester synthesis in organic solvents. This scheme offers an easy way to perform fermentation processes with substrate at high concentrations without product inhibition while promoting *in situ* its extraction as flavor compound, such as, ethyl butyrate.

EXPERIMENTAL WORK ON LIPASES

The earlier work carried out in our laboratory was related to the application of lipases in food industry (interesterification of canola oil). The purpose of this work was to identify reactor configurations and operating conditions suitable for the continuous interesterification of canola oil with a highly saturated fat, using immobilized lipase. This interesterification reaction resulted in the formation of fats suitable for the production of margarine and shortening, which have appropriate melting characteristics, polymorphic crystalline stability with reduced crystal size, and chemical composition[63].

More recently, a research project was developed for the optimization of a suitable processes for the industrial production of natural terpene esters for its use in flavor and fragrance applications. Terpene esters are very important aroma compounds. Currently, most of the flavor components are provided by traditional methods that include chemical synthesis or extraction from natural sources[87]. With the high demand for natural products, flavor industry is more interested in the use of biotechnology to produce natural flavors[24]. In this context, it is not surprising that many research efforts have been dedicated to the use of lipases for the production of flavor esters[89-92].

Research background on the production of terpene esters

Research interest on the application of lipases for production of terpene esters can be traced back to the early 1980's, in two papers by a Japanese group[93,94]. The most interesting aspect of these studies was the reaction conditions. The synthesis was carried out in an aqueous solutions consisting of 100μL of fatty acids, 200μL of terpene alcohols, 100μL (100 units of activity) of lipase solution (from *Aspergillus niger*) and 1000μL of H_2O[93]. As can be expected, very low product yields were reported (Table 5), since the thermodynamic of ester synthesis is unfavorable at high water concentrations.

Over the last few years the feasibility of such process has been completely re-evaluated by exploring the ability of enzymes to work on organic solvents. In such systems lipases remain active and can catalyze a wide range of esterification and transesterification reactions. High yields of products can be obtained since restricted amounts of water reduces the normal hydrolytic action of these enzyme preparations.

Several papers dealing with this subject have appeared since then in the literature[95-104]. As far as terpene alcohol esterifications are concerned, two research groups that have really contributed in this field are mentioned here as a way of illustration. The first is Tanaka's group in Japan and the other group is Barrati's group in France. A summary of their works is listed in tables 6 and 7.

Table 5. Product yields reported for several esterification reactions carried out in aqueous media.

Terpene Alcohol	Synthesis (%)
Geraniol	40
dl- Citronellol	33
l- Menthol	0
Linalool	0
l- Terpineol	0
Farnesol	17
Phytol	5

Citronellyl butyrate as a model of study

Our aim was to develop and optimize processes suitable for the industrial production of "natural" and novel terpene esters for its use in flavour and fragrance applications. Such processes made use of existing commercial lipase preparations in organic solvents under low water concentrations conditions to carry out esterification and transesterification reactions on terpene alcohol starting compounds.

The enzyme used in all the experiments was a commercial lipase preparation supplied by Novo Nordisk-Lipozyme IM 20 which is a fungal lipase from *Mucor miehi*, immobilized on a macroporous synthetic resin. Lipozyme is supplied with approx. 10%w/w water. If variations on moisture content were observed, original water content could be restored by either drying or hydrating the enzyme. The activity of Lipozyme depends on the reaction temperature being maximum at 70°C. However, for long-term operations, it is recommended to use it at 60°C, as the slightly lower activity at 60°C is counterbalanced by an equally slower deactivation of the enzyme[57]. Besides this, Novo's Lipozyme has been showed to have a high water binding capacity[55,56] which allows its use in a single run without any interference on the desirable reaction direction. The content of Lipozyme is said to be the most powerful factor on the reaction equilibrium position by keeping low water levels throughout the esterification reaction. The most important characteristics of Lipozyme are summarized in table 8.

Following the general rule that high biocatalyst activity is attained in relatively hydrophobic solvents and none or low activity is observed in relatively hydrophilic solvents[26], heptane was chosen as the solvent medium. Besides having logP = 4.0 that is supposed to give good stability for the enzyme system, the results available in the literature provide a basis for comparison with our own data[98-100].

As starting materials, the most important terpene alcohols (geraniol, nerol, linalool and citronellol) used in fragrance and flavors substances[67] were employed to check the ability of the selected enzyme to perform ester synthesis with butyric acid as acyl donor. Except linalool, which has a different chemical structure, the enzyme performed all the esterification reactions with satisfactory yields. Since citronellol represents the most widely used fragrance material[106] particularly for roses notes and for floral compositions in general and is the starting material for many citronellyl esters, the system Lipozyme/Citronellol/Butyric Acid/Heptane was chosen as a model to study the kinetic parameters and role of water in the production of terpene esters (Figure 8).

Table 6. Summary of the work reported by Tanaka's research group.

Terpene Alcohol	Acyl donor	Lipase source	Solvent	Reaction Conditions	Results/Comments
Citronellol	5 phenyl valeric acid	37 kinds of lipases	cyclohexane isooctane	30°C 120rpm 93h	22 lipases had a high activity in cyclohexane
130 mM	100 mM	100 mg	10 mL		Immobilization in hydrophobic gels enhanced the operational stability of the lipase in organic media
Menthol borneol citronellol linalool neurodiol terpineol	5 phenyl valeric acid	*Candida cylindracea* entrapped in urethane	cyclohexane	30°C 120rpm 93h	The enzyme activity for preparation of terpene alcohol can be summarized as:
	100 mM	prepolymers	100 mL		
					1.Alcohol: primary> secondary > tertiary
130 mM		100 mg			
					2.Acyl donor: short chain< middle chain<long chain
					3.Temperature: low < high

Other major contributions includes:
Development of immobilized lipase preparation which retained more than 90% of the original activity after running 12 batches (each batch/ 24 hours). The immobilization consists in entrapment of a celite-absorbed enzyme with hydrophobic photocross linkable resin prepolymer ENTP - 2000.

Table 7. Summary of the work reported by Baratti's research group.

Terpene Alcohol	Acyl donor	Lipase source	Solvent	Reaction Conditions	Results/ Comments
citronellol geraniol	acetic, butyric, valeric,	*Mucor miehi, Aspergillus sp, C. rugosa*	heptane (5 mL) and	37°C 18h reciprocal	Conversion yields varied markedly with the lipase preparation, the nature of acid
0.25 M	caproic acids	*R. arrhizus*	water	agitation	and alcohol moiety of the ester. Better conversions were achieved with lipases from
	0.25 M	(0.25 g)	(0.2%)		*M.miehi* and *R.arrhizus* (<70%)
geraniol	acetic, butyric	13 different sources of	heptane (10 mL)	37°C 24h	6 lipases, including Lipozyme, yielded more than 90% for
0.25 M	acids	lipases, including	and water	reciprocal agitation	geranyl butyrates. Most of the lipases were not able to catalyze
	0.25 M	Lipozyme	(0.1%)		the synthesis of geranyl acetates. 4 lipases showed yields of geranyl
		(0.50 g)			propionates higher than 50%
menthol	butyric acid	*Candida rugosa*	heptane (20 mL) and	40°C stirring magnetic bar	Since butyric acid is a good acyl donor and its concentration is higher compared with menthol,
0.5 M	1.0 M		water (0.2 %)		ester formation was strongly favoured (conversion = 93%)

Other major contribution includes:
Development of kinetic model based on competitive reversible enzymatic reactions, taking into account the acyl transfer from an ester (an acid) to the enzyme or from the acyl enzyme to an acceptor compound.

Summary of the achieved results

In the preceding section we described the experimental methodology adopted in our laboratory for evaluating the selected biotransformation system. This has been used to determine several parameters on the reaction rate, enzyme stability, suitability of the solvent used and the influence of water on the esterification performance.

The results demonstrated the feasibility of the production of citronellyl butyrate from citronellol and butyric acid using Novo's Lipozyme as catalyst. High conversions were obtained despite no provision of water removal was performed[107,108]. This was associated with to the high hidrophilicity value of this enzyme preparation which absorbed the water formed throughout the reaction. The immediately advantage of such feature is to buffer the water activity of the reaction system so that the required synthesis direction is not inhibited. Consequently, there is significant change in the original hydration state of the enzyme which was found to be a limiting factor in maintaining high conversions in repeated batch use of the Lipozyme[106].

Table 8. Lipozyme properties according to the data provided by the manufacturer.

Microorganism	*Mucor miehi*
Carrier	Macroporous anion exchanger
Method	Ionic binding and adsorption
Particle size	300-600 μm grains
Pore radius	50 - 300 Å
Surface area	200 m^2/g
Water content	10%(w/w)
Molecular weight	32.000(SDS - PAGE)
Isoeletric point	4
Specificity fatty acid:	Broad
glyceride:	1,3 - positions
alcohol:	Primary - OH
pH - optimum	6-8
Temperature	60 - 70°C
Activity*	24 BIU

*One Batch Interesterification Unit (1BIU) is defined as 1 μmol of incorporated palmitic acid into triolein per minute at standart conditions.

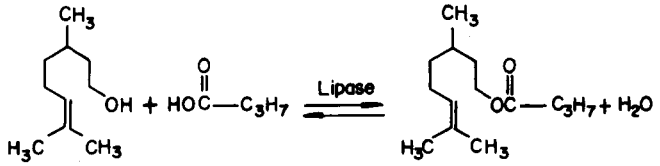

Figure 8. Esterification of citronellol with butyric acid catalyzed by lipase in heptane.

This problem was overcome by applying dehydration techniques between batches[58,108]. The use of molecular sieves was found to be a suitable dehydration technique, although a decrease of 30% occurred after 15 batch runs[58]. This decrease appears to be related not only with the water formed but also with the main reaction product (ester). Both of the products can inhibit to a certain extent the synthesis reaction direction. Solvents with high log P values, namely n-heptane, were found to be suitable for extracting compounds absorbed in the solid enzyme phase. This was considered to be a potential solution to overcome the problems associated with the repeated use of Lipozyme[108,109]. A summary of these results are listed in table 9.

Table 9. Summary of the achieved results using citronellyl butyrate as a model system.

Tested Parameters	Established Conditions
Solvent	Heptane
Water concentration	
liquid phase	100-200 ppm
solid phase	0.1g H_2O/g of enzyme
Molar ratio (butyric acid: citronellol)	greater than 1
Temperature	60°C
Enzyme concentration	1 gram of Lipozyme/ mol of citronellol
Water removal during single batch runs	Procedure unnecessary
Repeated batch use of Lipozyme	Feasible when Lipozyme was dehydrated after each batch
Dehydration technique	Molecular sieves or solvent washing

All the laboratory work so far completed has been based on the use of lipase for esterification reactions. Development is now focused on the application of lipase for the resolution of racemic alcohols and carboxylic acids through asymmetric hydrolyses of the corresponding esters. For this purpose, an experimental protocol was set up in which lipase was used to convert a racemic citronellyl mixture to (R)-citronellol, a preferred component in various perfumes. This is a straightforward procedure for the resolution of several racemic mixture[8,9] and our aim was to develop a procedure for selective reaction to yield the alcohol of choice with high optical purity. This work is still under development and the results will be further reported.

FUTURE PROSPECTS

Lipase is a group of enzymes which under aqueous conditions hydrolyze ester bonds of triglycerides. In recent years commercial interest in lipase has increased because their mode of action can be reversed in organic media so that they catalyze ester synthesis or transesterifications.

The aim of this paper was to give a view to intensity the interralions of chemistry and biotechnology. By improving the knowledge of chemists in the field of biotechnology it is hoped to initiate more ideas for applying biological methods in organic synthesis, inspire more use of chemical knowledge in the biological sciences and help scientist in both fields to work close together.

As a practical example of such interactions, we have described experimental tools for the characterizing biotransformations with lipases. This has been illustrated by using several industrial processes. These techniques have also been applied successfully in our laboratory to carry out the esterification of terpene alcohols by a commercial immobilized lipase preparation. This allowed the various parameters to be determined and access strategies for the enzyme activity regeneration under repeated batch runs. The emphasis has now shifted to optimize the resolution of optical isomers of citronellyl butyrate by hydrolysis and interesterification reactions where there is no water formation throughout the process. With this work, we are now expecting to extend our understanding to the complex evaluation of the biotransformation reactions catalyzed by lipases.

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