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APPLICATION OF LIPOLYTIC ENZYMES OF MICROBIAL ORIGIN AS BIOCATALYSTS

Doctoral (Ph.D) Dissertation

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TABLE OF CONTENTS

Abbreviations

Abbreviations	Original expressions /terms
*Ao*SSF	*Aspergillus oryzae* originated from Solid State Fermentation
*As*SSF	*Aspergillus sojae* originated from Solid State Fermentation
BRIJ	BRIJ 30
c	Conversion
DAG	Diacylglycerol
DCC	Dicyclohexylcarbodiimide
DecTEOS	Decyltriethoxysilane
DMAP	4-Dimethylaminopyridine
DMDEOS	Dimethyldiethoxysilane
DodTEOS	Dodecyltriethoxysilane
E	Enantiomer selectivity
ECN	Equivalent carbon number
ee	Enantiomeric excess
f	Flow rate
FA	Fatty acid
GC	Gas chromatography
HexTEOS	Hexyltriethoxysilane
HPLC	High performance liquid chromatography
K_m	Michaelis-Menten constant
LA	Lauric acid
LCFA	Long chain fatty acid
LLM	Triglycerides composed of Long – Long – Medium chain fatty acids
LLL	Triglycerides composed of Long –Long–Long chain fatty acids
LML	Triglycerides composed of Long –Medium–Long chain fatty acids
L-TEG-L	Tetraethylene glycol dilauryl ester
MCFA	Medium chain fatty acid
MCT	Medium chain triglyceride
MML	Triglycerides composed of Medium – Medium– Long chain fatty acids
MLM	Triglycerides composed of Medium – Long – Medium chain fatty acids
MUFA	Monounsaturated fatty acid
OA	Oleic acid

Oc(t)TEOS	Octyltriethoxysilane
OctdTEOS	Octadecyltriethoxysilane
P	Product
PEG400/PEG1000	Polyethylene glycol
PFOctTEOS	Perfluorooctyltriethoxysilane
PhTEOS	Phenyltriethoxysilane
PrTEOS	Propyltriethoxysilane
PS	Phytosterol (plant sterol)
PSE	Phytosterol ester
PUFA	Polyunsaturated fatty acid
r	Specific reaction rate (productivity)
S	Substrate
SCFA	Short chain fatty acid
SEM	Scanning electron microscopy
SFA	Saturated fatty acid
SL	Structured lipid
SSL	Specific structured lipid
TEG	Tetraethylene glycol
TEOS	Tetraethoxysilane
TLC	Thin layer chromatography
TRX100	Triton X-100
U_B	Specific biocatalyst activity
Y_A	Activity yield

1. INTRODUCTION

Biocatalysis has emerged as an important tool in the industrial synthesis of bulk chemicals, pharmaceutical and agrochemical intermediates, active pharmaceuticals, and food ingredients. However, the number and diversity of the applications are modest, perhaps in part because of perceived or real limitations of biocatalysts, such as limited enzyme availability, substrate scope, and operational stability. Recent scientific breakthroughs in genomics, directed enzyme evolution and the exploitation of biodiversity should help to overcome these limitations.

Worldwide, chemists are vigorously taking on the challenge of developing synthetic methodology and „green" processes that meet the criteria of a sustainable, environmentally conscious development. The biocatalytic transformations today are routinely considered by synthetic organic chemists, and by process engineers, as an economically and ecologically competitive technology, and as a matter of fact for the development of new production routes to fine chemicals, pharmaceuticals, agrochemicals, and even bulk commodities. Market demands are now pushing for individual tailoring of biocatalysts for specialized applications, driven by the new opportunities that have emerged for the discovery of novel enzymes and the fine-tuning, and even redesign of their properties.

Industry continues to demand more selective and efficient catalysts and processes for the manufacture of fine chemicals. Here, enzyme catalysts often have a „natural" advantage that will increasingly be exploited as the demand for enantiomerically pure drugs continues to rise. High economical and low ecological impact, it is adopted for industrial large-scale conversions.

Hydrolases predominate for several obvious reasons, the range of enzyme classes considered for technical processes is growing remarkably broad, and a steadily increasing number of different bioprocesses are being successfully transplanted from the laboratory to the manufacturing plant. To a large degree, this growth is a result of improved access to stable biocatalysts having customized activities and selectivities. This reflects the recent progress in the fields of molecular biology, high-throughput screening techniques, and advanced engineering (FESSNER and JONES 2001).

The use of biocatalysis for industrial synthetic chemistry is on the way of significant growth. Biocatalytic processes can now be carried out in organic solvents as well as aqueous environments, so that apolar organic compounds as well as water-soluble compounds can be modified selectively and efficiently with enzymes and biocatalytically active cells. As the use of biocatalysis for industrial chemical synthesis becomes easier, several chemical companies have begun to increase significantly the number and sophistication of the biocatalytic processes used in their synthesis operations (SCHMID et al., 2001).

The application of biocatalysts offers a remarkable arsenal of highly selective transformations for modern preparative organic chemistry. Biocatalysis can provide environmentally friendly processes for all life science related industries (pharma, food, feed, agro).

The development of technologies using lipases for the synthesis of novel compounds will result in their expansion into new areas and increase in number of industrial applications

The economic synthesis of the increasing number of biologically active molecules is one of the biggest challenges on the field of organic chemistry. The preparation and the use of large purity enantiomers are obviously necessary for different industries (mainly pharmaceutical, plastic, cosmetic and food industry) (POPPE and NOVÁK 1991, 1992; BULL et al. 1999, FABER 2004). The presence of the other enantiomer besides the active one is a real danger in effective drugs. The most known example for that is the (*R*)-Thalidomide (α-phtalimido-glutaramide, *Contergan*), which has a sedative effect, while its enantiomer pair, the (*S*)-Thalidomide is teratogenic even in small amount (NISHIMURA and TANIMURA 1976).

Besides the traditional chemical methods, the growth of the biocatalytical methods both in laboratory and industrial scale are occurred due to environmental aspects and the increased demand toward stereoselective synthesis. As a result of this process the world leading pharmaceutical companies are spending huge amount of money to research projects finding solution to these questions.

The chemical industry at the end of the 20^{th} century was developing much slower and adopted novel technologies less effectively than other industries. The underlying problem is that chemists are working on a fixed, batch based infrastructure, especially at research and development. As a result the scale up is problematic and time consuming resulting in further deviation from environmental and economic efficiency. Continuous processes could provide essential solutions to this problem; however integrated flow technologies that could cover from lab scale to pilot and production scale exist only scattered today. There are only a few examples of hydrolase-catalyzed enantioselective processes carried out in continuous-flow systems (PATEL et al. 1996, CHEN and TSAI 2000, UJANG et al. 2003). Although the immobilized biocatalysts can be utilized ideally in continuous-flow systems, most of the continuous-mode biocatalytic syntheses of chiral pharmaceutical intermediates were performed on a relatively large scale using immobilized lipases in a packed-bed reactor so far (PATEL et al. 1996, LIESE et al. 2006). It was found that stainless-steel continuous-flow packed-bed bioreactors could be effectively used to study the effects of temperature, pressure and flow rate on lipase-catalyzed kinetic resolution (CSAJÁGI et al. 2008, TOMIN et al. 2010a).

In the 21st century mankind has to battle /be taken the field against chronic diseases such as obesity, high blood pressure and cholesterol level, cardiovascular diseases, diabetes, colon cancer etc. Certainly it can be stated that besides genetic factors and lifestyles nutrition and diet play an important role in evolving diseases. Thus the main focus has to be taken rather on prevention than on the treatments of the already existing diseases.

Nowadays, functional foods which have beneficial physiological effects on human body and/or can reduce the risk of chronic diseases, have an emerging role.

Food industry is also facing new challenges due to rapid growth of market for functional and nutraceutical foods and consumer demand for more healthy fats and has to turn toward the implementation of mild processes. Application of biocatalysis methods can be a proper tool to provide solution for these challenges.

2. LITERATURE OVERVIEW

2.1. Biotransformations

Throughout the history of mankind, microorganisms had enormous social and economic importance. Very early in the history man was using them in the production of food products such as cheese, sourdough, beer, wine and vinegar, and in the manufacture of commodities such as leather, indigo and linen. All of these processes relied on either enzymes produced by spontaneously growing microorganisms or enzymes present in added preparations such as calves' rumen or papaya fruit. The Sumerians and Babylonians were practicing the brewing of beer before 6000 BC, references to wine making can be found in the Book of Genesis and the Egyptians used yeast for baking bread. However the knowledge of the production of chemicals such as alcohols and organic acids through fermentation is relatively recent and the first reports in the literature only appeared in the second half of the 19th century. Lactic acid was probably the first optically active compound to be produced industrially by fermentation. In the course of time it was discovered that microorganisms could modify certain compounds by simple, chemically well-defined reactions which were further catalyzed by enzymes. Nowadays, these processes are called biotransformations. The essential difference between fermentation and biotransformation is that there are several catalytic steps between the substrate and the product in fermentation while there is only one or two in a biotransformation. The distinction is also in the fact that the chemical structures of the substrate and the product resemble one another in a biotransformation, but not necessarily in fermentation (LIESE et al. 2006, KIRK et al. 2002).

Over the past few decades, major advances in our understanding of the protein structure-function relationship have increased the range of available biocatalytic applications. In particular, new developments in protein design tools such as rational design and directed evolution have enabled scientists to rapidly tailor the properties of biocatalysts for particular chemical processes.

Enzyme properties such as stability, activity, selectivity and substrate specificity can be routinely engineered in the laboratory. Presently, approximately 100 different biocatalytic processes are implemented in pharmaceutical, chemical, agricultural and food industries. The products range from research chemicals to commodity chemicals and the number of applications continue to grow very rapidly. In spite of theses successes, the vast potential of biocatalysis has not been fully realized (JOHANNES et al. 2006).

2.2. Enzymes in general

"Enzyme" was used as the new term to denote phenomena previously known as "unorganized ferments", which was isolated from the viable organisms in which they were formed. The word literally means "in yeast" and is derived from the Greek "en" (meaning "in"), and "zyme" (meaning "yeast" or "leaven". In living organisms most of the reactions are catalyzed by protein molecules called enzymes which are the catalytic machinery of living systems. Man has indirectly used enzymes almost since the beginning of human history. Enzymes are responsible for the biocatalytic fermentation of sugar to ethanol by yeasts, a reaction that forms the bases of beer and wine manufacturing. Enzymes oxidize ethanol to acetic acid. This reaction has been used in vinegar production for thousands of years. The brief history of enzymes and their applications can be found in Table 1 (DRAUZ and WALDMANN 2002, CAO 2005a).

2.2.1. History

Table 1. Brief history of enzymes and their applications (DRAUZ and WALDMANN 2002)

Date	Discovery and application of enzymes	References
B.C	Chymosin from the stomach of young cattle, sheeps and goats was used for cheese production in many ancient cultures for approximately 7000 years.	
1783	Hydrolysis of meat by gastric juice demonstrated.	Spallazani
1814	Starch degradation and sugar production by malted barley observed.	Kirchhoff
1833	The active principle of malt is called diastase and its application to industrial art described.	Payen and Persoz
1846	Invertase activity observed.	Dubonfout
1867	The term *enzyme is* coined to describe catalytic activity not bound to living cells (unorganized ferments). The name is extended later also to intracellular catalysts (organized ferments as defined by Pasteur).	Kühne
1893	Definition of catalyst including enzymes is given.	W. Ostwald
1894	Enzyme stereospecificity anticipated. "Lock and key" concept.	E. Fischer
1894	"Taka diastase" produced commercially with *Aspergillus oryzae* by surface culture.	Takamine
1897	The conversion of glucose to ethanol demonstrated by a cell free extract from yeast.	E. Buchner
1906	Preparative separation of L-leucine from the racemate carried out by hydrolysis of the propyl ester with liver extracts.	Warburg
1908	Synthesis of optically active cyanohydrins described, using D-oxynirerilase from almonds as catalyst.	Rosenberg
1908	Application of pancreatic enzymes in the leather industry for the bating of hides.	O. Röhm
1911-1913	Glucoside synthesis in the presence of high concentration of ethanol or acetone described.	Bourquelot, Bridel and Verdon
1913-1915	Application of pancreatic enzymes to clean laundry introduced, first commercial product sold	Röhm Company
1916	Immobilization of invertase on charcoal demonstrated with retention of activity.	Nelson and Griffin
1926	Urease from Jack beans crystallized.	J. B. Summer
1936	Enzymatic ester synthesis improved using pancreatic lipase in the presence of benzene.	E. Sym
1953	The first primary sequence of a protein (insulin) established, proving the chemical identity of proteins.	F. Sanger, H.Tuppy
1960	Cultivation of *Bacillus licheniformis* in submerged culture started for protease production on large scale.	Novo
1980 –	Application of genetic engineering techniques to improve enzyme production and to alter enzyme properties by protein engineering and evolutionary design.	many…

2.2.2. Catalysis

In catalytic reactions catalysts are accelerating the rate of reaction, usually present in small managed quantities and unaffected at the end of the reaction. They are used to lower the activation energy of a reaction compared to the corresponding uncatalyzed reaction thus resulting in higher reaction rate at the same temperature. This way catalyst permits reactions or processes to take place more effectively or under milder conditions than would otherwise be possible. Unlike other reagents that participate in the chemical reaction, a catalyst is not consumed by the reaction itself.

Catalysis is a key technology to provide realistic solutions to many environmental issues and to promote sustainability, environment, energy, health and quality of life. As with the application of more selective and more accelerated reaction pathways can be worked out and formation of wastes can be avoided.

2.2.3. Enzymes as catalysts

Chemical reactions are far too slow to be effective under normal living systems conditions such as aqueous environments with neutral pH and temperature between 20-40°C. In comparison enzymes can achieve up to 10^7 –fold faster reaction rates than the catalysts developed by chemical industry. As catalysts the enzymes alter the rate at which thermodynamic equilibrium is reached, but do not change the equilibrium. This implies that enzymes work reversibly. The acceleration in reaction rate is achieved by lowering the activation energy of the overall process as shown schematically in Figure 1.

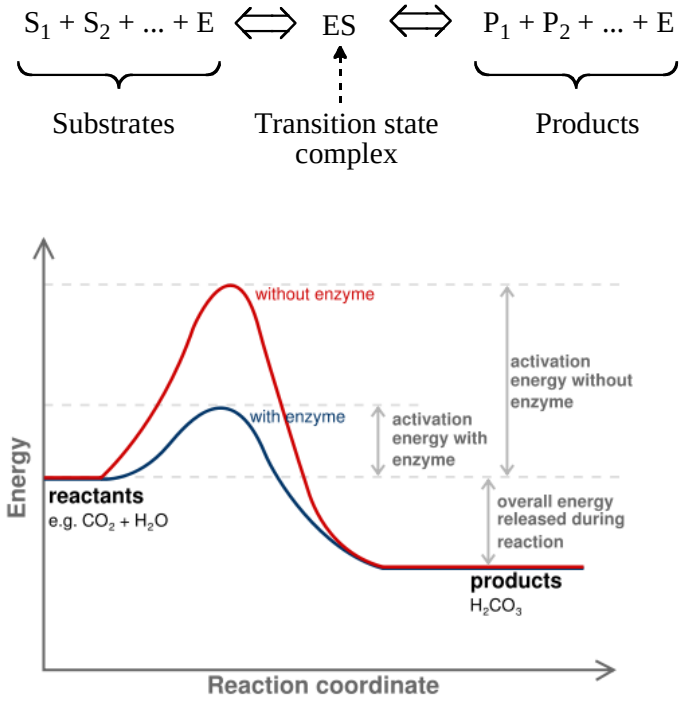


Figure 1. General principle of enzyme catalysis

The reason why synthetic chemists have become interested in biocatalysis is mainly due to the need to synthesize enantiopure compounds as chiral building blocks for drugs and agrochemicals. Another important advantage of biocatalysts are that they are environmentally acceptable, being completely degraded in the environment. Furthermore the enzymes act under mild conditions, which minimizes problems of undesired side-reactions such as decomposition, isomerization, racemization and rearrangement, which often plague traditional methodology, simply separation process, higher yield and due to mild reaction conditions, energy and waste treatment costs saving.

2.2.4. Advantages and disadvantages of biocatalysis vs. chemical catalysis

Similar to other catalysts, biocatalysts increase the speed in which reaction takes part but do not affect the thermodynamics of the reaction. However, they offer some unique characteristics over conventional catalysts. The most important advantage of biocatalysts is its high selectivity. This selectivity is often chiral (stereoselectivity), positional (regioselectivity) and functional group specific (chemoselectivity). Such high selectivity is very desirable in chemical synthesis as it may offer several benefits such as reduced or no use of protecting groups, minimized side-reactions, easier separation and fewer environmental problems. Other advantages, like high catalytic efficiency and mild operational conditions are also very attractive in commercial applications.

The characteristics of limited operating regions, substrate or product inhibition and reactions in aqueous solutions have often been considered as the most serious drawbacks of biocatalysts. However it was turn out about these drawbacks to be microconceptions. For example, many commercially available enzymes show excellent stability under process conditions. In addition there is an enzyme-catalyzed reaction, equivalent to almost every type of known organic reaction. Many enzymes can accept non-natural substrates and convert them into desired products. Almost all of the biocatalyst characteristics can be tailored or rationally designed to meet the desired process conditions (JOHANNES et al. 2006).

2.2.5. Enzyme structure and function

All enzymes are proteins, with the exception of the recently discovered ribozymes. Proteins are linear polymers defined by the amino acids sequence (*primary structure*) linked by peptide bonds.

To generate a specific surface as part of the active center of an enzyme the protein chain has to be fold. Two structural arrangements of polypeptide become energetically favored, the α–helix and β–pleated sheet which are further stabilized by H-bonds between the peptide backbone. Helices and plated sheets are commonly found in proteins called *secondary structure* and when these secondary structure elements are linked by loops to build a domain or a subunit, this level of organization is

the *tertiary structure*, when more subunits are connected into homo- or hetero-oligomers is called *quaternary structure*. The folded structure of a protein is stabilized by a network of non-covalent interactions such as hydrogen-bonds, disulfide bonds, hydrophobic interactions, ionic bonds, salt bridges and van der Waals interactions.

The chemical potential of side chains found in amino acids is limited, there are no efficient electron acceptors, therefore, and it requires additional chemical potential by specific *metal ions* (Zn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}). Besides metal ions *cofactors* or *coenzymes* serve to activate groups and participate in the catalytic process. Cofactors (sometimes called prostetic groups) are covalently bound to the protein and may undergo cyclic reactions during catalytic process but will return to the ground state at the end. Coenzymes are bound in association-dissociation equilibrium to enzymes and have to be present in sufficient concentration to obtain maximal enzymatic activity. Some are regenerated in the catalytic cycle while bound to the enzyme. In such instances, the enzyme without the cofactor is called an *apoenzyme*, and the apoenzyme-cofactor complex is called a *holoenzyme* (DRAUZ and WALDMANN 2002).

2.2.6. Enzyme applications in the industries

Enzymes are used in a wide range of industries. They are required only in small quantities to synthesize kilograms of stereochemically challenging chiral materials that are used as building blocks to produce highly active pharmaceuticals. Their versatility allows their use in many applications, including processes to degrade natural polymers such as starch, cellulose and proteins, as well as for the regioselective or enantioselective synthesis of asymmetric chemicals. The latest developments within biotechnology, introducing protein engineering and directed evolution, have further revolutionized the development of industrial enzymes (KIRK et al. 2002, LORENZ and ECK 2005).

Table 2. Enzymes used in various industrial segments and their application (KIRK et al. 2002)

Industry	Enzyme class	Application
Detergent (laundry and dish wash)	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, color clarification, anti-redeposition (cotton)
	Mannanase	Mannanan stain removal (reappearing stains)
Starch and fuel	Amylase	Starch liquefaction and saccharification
	Amyliglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerase	Glucose to fructose conversion
	Cyclodextrin-glycosyltransferase	Cyclodextrin production
	Xylanase	Viscosity reduction (fuel and starch)
	Protease	Protease (yeast nutrition –fuel)
Food (including diary)	Protease	Milk clotting, infant formulas (low allergenic), flavor
	Lipase	Cheese flavor
	Lactase	Lactose removal (milk)
	Pectin methyl esterase	Firming fruit-based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume, flour adjustment
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning (*in situ* emulsifier)
	Phospholipase	Dough stability and conditioning (*in situ* emulsifier)
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strengths
Animal feed	Phytase	Phytate digestibility –phophorous release
	Xylanase	Digestibility
	β-Glucanase	Digestibility
Beverage	Pectinase	Depectinization, mashing
	Amylase	Juice treatment, low calorie beer
	β-Glucanase	Mashing
	Acetolactate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavor (beer), cork stopper treatment
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	Desizing
	Pectate lyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch coating, deinking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	Deinking, drainage improvement, fiber modification
Fats and oils	Lipase	Transesterification
	Phospholipase	Degumming, lysolecithin production
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semisynthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxylic acids
Leather	Protease	Unhearing, bating
	Lipase	Depickling
Personal care	Amyloglucosidase	Antimicrobial (combined with glucose oxidase)
	Glucose oxidase	Bleaching, antimicrobial
	Peroxidase	Antimicrobial

There is a global drive to promote white (industrial) biotechnology. This requires the development of novel enzymes, processes, products and applications. For any industrial application, enzymes need to function sufficiently well according to several application-specific parameters. Figure 2 illustrates the ideal biocatalyst concept. Each enzyme candidate is ranked, from low (rating

to 1) to high (rating of 6) using a specific set of criteria, to produce a multi-parameter fingerprint. Criteria include enzyme activity, efficiency, specificity and stability (LORENZ and ECK 2005).

From economical and technological point of view those enzymes are preferred which do not require cofactor for their operation (hydrolases, lyases and most of the isomerases) otherwise cofactor requirement can cause regeneration problems and extra expenses.

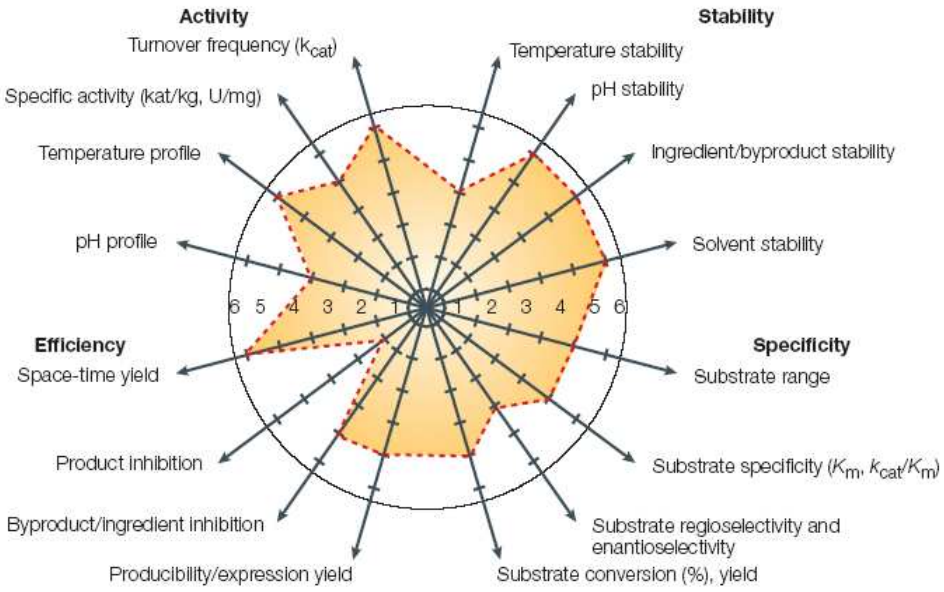


Figure 2. Multi-parameter footprint analysis (*kat*, catalytic reaction rate; K_{cat}, catalytic constant, K_m, Michaelis-Menten constant; U, Unit.) (LORENZ and ECK 2005)

2.2.7. Selectivity

The key word for organic synthesis is selectivity, which is necessary to obtain a high yield of a specific product. There are a large range of selective organic reactions available for most synthetic needs. However, there is still one area where organic chemists are struggling, and that is when chirality is involved, although considerable progress in chiral synthesis has been achieved in recent years. The selectivity of enzymes nowadays becoming a powerful asset of enzyme mediated asymmetric synthesis, because of the increasing need of the pharmaceutical industry for optically pure intermediates.

Enzymes display four major types of selectivities:

In general the selectivity of enzymes includes *substrate selectivity* (the ability to distinguish and act on a subset of compounds within a larger group of chemically related compounds) *stereoselectivity*, (the ability to act on a single enantiomer or diastereomer exclusively), *regioselectivity* (the ability to act exclusively on one location in a molecule), functional group selectivity (the ability to act on one *functional group selectively* in the presence of other equally reactive or more reactive functional groups).

Here two additional important selectivity related terms have to be introduced for the characterization of enzyme catalyzed enantioselective reactions, one of them is *enantiomeric excess* (*ee*) and the other is *enantiomer selectivity* (*E*) (GUO and SIH 1989).

Enantiomer excess

The enantiomeric excess (ee) is the difference between the numbers of both enantiomers per sum of the enantiomers:

$$ee[\%] = \frac{M - M^*}{M + M^*} \times 100 \qquad \text{(Equation 1)}$$

where M is the molar fraction of main enantiomer, M^* is the molar fraction of contaminant enantiomer.

The enantiomeric excess describes the enantiomeric purity of an optically active molecule. It is essential to choose biotransformation processes that lead to products with high enantiomeric excess. This is because the pharmacological activities of the enantiomers might not be identical. Each enantiomer could even have a completely different pharmacological activity profile, including serious side effects in certain case. The enantiomers often have different organoleptic properties such as taste, flavor or odor. Many pharmaceuticals and agrochemicals, which were previously sold as racemates, are now sold as single enantiomer products ("racemic switches"). The US FDA (Food and Drug Administration) makes it mandatory for drug companies to carry out clinical trials of the individual enantiomers before selection of the correct enantiomer as the active pharmaceutical ingredient. The use of enantiomerically pure drugs instead of racemates avoids the intake of inactive or even toxic compounds. Similarly, this applies to the use of enantiomerically pure agrochemicals (LIESE et al. 2006).

Enantiomer selectivity

Hydrolases reacting with a racemic substrate normally display a preference for reacting faster with one enantiomer of the substrate e.g. the (*R)*-enantiomer than with the (*S)*-one. The initial relative rate of reaction for the enantiomers of a racemic substrate is called enantiomeric ratio or the E-value and is defined as the ratio between the specificity constants for the two competing enantiomers. When the enantiomeric excess of one of either the substrate or the product, ee_S and ee_P, respectively, and the conversion are known, the E-value of the reaction can be calculated from Equation 2 for reversible reactions and Equations 4-6 for irreversible ones.

The efficiency of distinguishing the two competing enantiomers can be characterized by *E* value, which in case of reverse reactions can be calculated as follows (CHEN et al. 1982):

$$E = \frac{\ln[1-(1+K)c(1+ee_P)]}{\ln[1-(1+K)c(1-ee_P)]} = \frac{\ln[1-(1+K)(c+ee_S\{1-c\})]}{\ln[1-(1+K)(c-ee_S\{1-c\})]} \qquad \text{(Equation 2)}$$

In this equation ee_P referring to the product while ee_S refers to the initial enantiomer excess. K is the equilibrium constant, while conversion (*c*) can be defined as follows:

$$c = \frac{ee_S}{ee_S + ee_P} \qquad \text{(Equation 3)}$$

The formula or equation can be extensively simplified in case of irreversible reactions. There are three ways/options to calculate enantiomer selectivity (*E*) from the conversion (c) and the values of enantiomeric excess of product and the fractions of substrates respectively (ee_P, ee_S)

from ***c*** and $\mathbf{ee_P}$ values (CHEN et al. 1982):

$$E_{c,ee_P} = \frac{\ln[1-c(1+ee_P)]}{\ln[1-c(1-ee_P)]} \qquad \text{(Equation 4)}$$

from ***c*** and $\mathbf{ee_S}$ values (CHEN et al. 1982):

$$E_{c,ee_S} = \frac{\ln[1-c(1-ee_S)]}{\ln[1-c(1+ee_S)]} \qquad \text{(Equation 5)}$$

from $\mathbf{ee_S}$ and $\mathbf{ee_P}$ values (RAKELS et al. 1993):

$$E_{ee_S,ee_P} = \frac{\ln[(1-ee_S)/(1+ee_S/ee_P)]}{\ln[(1+ee_S)/(1+ee_S/ee_P)]} \qquad \text{(Equation 6)}$$

Normally E-value is independent of conversion however depends on the enzyme used and its preparation, the substrate, the solvent, the reaction temperature and other reaction conditions.
Graphs of the course of typical reactions are shown in Figure 3 A and B for reversible and irreversible reactions, respectively.

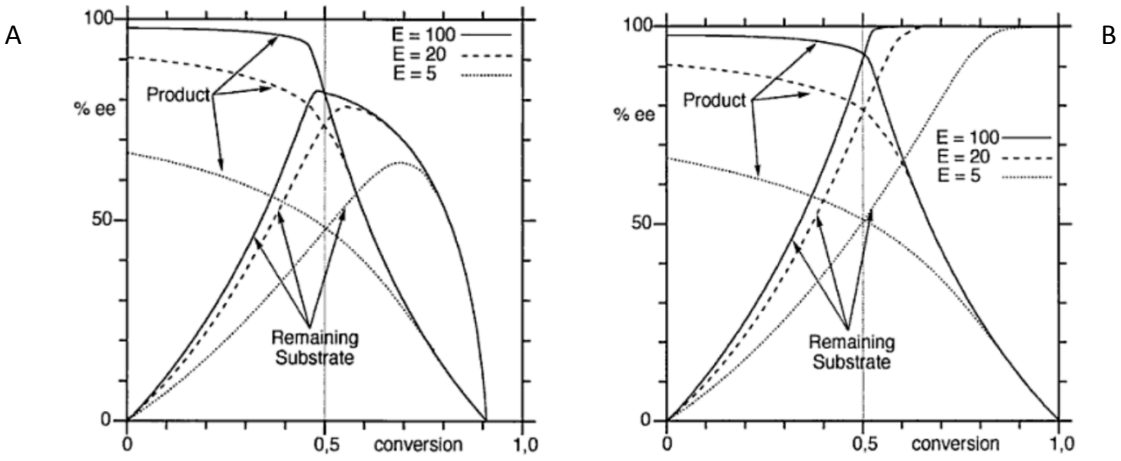


Figure 3. Reversible (A) and irreversible (B) reaction (CARREA G., 2008)

At low conversions (roughly below 40% conversion) the product curves for a given E-value are virtually identical for both the reversible and irreversible cases. For obtaining both the product and the remaining substrate in high enantiomeric excess in one reaction step, the E-value needs to be

high, usually around over 100. In case of irreversible reaction it is possible to obtain the remaining substrate in very high enantiomeric excess even when the reaction has a rather low E-value. The product cannot be obtained in a higher *ee* than that which is determined by the E-value. Thus at E=100 and at the start of the reaction, the first product formed has a maximum *ee* of 98% and *ee* then decreases with increasing conversion. In that case the remaining substrate can be obtained in 100% *ee* after approximately 55% conversion. While in case of reversible reactions neither the product nor the remaining substrate can be obtained in a very high *ee*. Thus the reversibility lowers the *ee*-value of remaining substrate at high conversions (CHEN et al. 1982, RAKELS et al 1993, CARREA and RIVA 2008).

2.2.8. Enzyme nomenclature

With the great progress achieved in the area of biochemistry in the 1950s' a large number of enzymes could be isolated and characterized. By this time it was necessary to regulate the enzyme nomenclature. Thus International Union of Biochemistry and Molecular Biology (IUBMB) in consultation with International Union for Pure and Applied Chemistry (IUPAC) set up an Enzyme Commission (EC) to be in charge of guiding the naming and establishing a systematic classification for enzymes (LIESE et al. 2006). According to the type of reaction catalyzed, the Enzyme Commission has classified the enzymes into 6 main classes:

1. Oxidoreductases (E.C.1…) catalyze oxidation/reduction reactions, transferring hydrogen, oxygen and/or electrons between molecules. In this important class belong dehydrogenases, oxidases, oxygenases and peroxidases.
2. Transferases (E.C.2…) catalyze the transfer of groups of atoms (amino-, acetyl-, phosphoryl-, glycosyl-etc.) from a donor to a suitable acceptor.
3. Hydrolases (E.C.3…) catalyze the hydrolytic cleavage of bonds. Many commercially important enzymes belong to this class, e.g. proteases, amylases, acylases, lipases and esterases.
4. Lyases (E.C.4…) catalyze the non-hydrolytic cleavage of for example C=C, C=O, C=N bonds by elimination reactions leaving double bonds or reverse adding groups to a double bond.
5. Isomerases (E.C.5…) catalyze isomerization and transfer reaction within one molecule. The most prominent member of this group is glucose isomerase or e.g. *Z-E* and *cis-trans* isomerization.
6. Ligases (E.C.6…) catalyze the covalent joining of two molecules coupled with the hydrolysis of an energy rich bond in ATP or similar triphosphates. Ligases find limited applications only for synthetic purposes.

The main classes are further subdivided into subclasses and subgroups.

With the rapid technical developments in gene discovery, optimization, and characterization, enzymes have been increasingly used as biocatalysts. Only a limited number of all the known enzymes are commercially available and even smaller amount is used in large quantities.

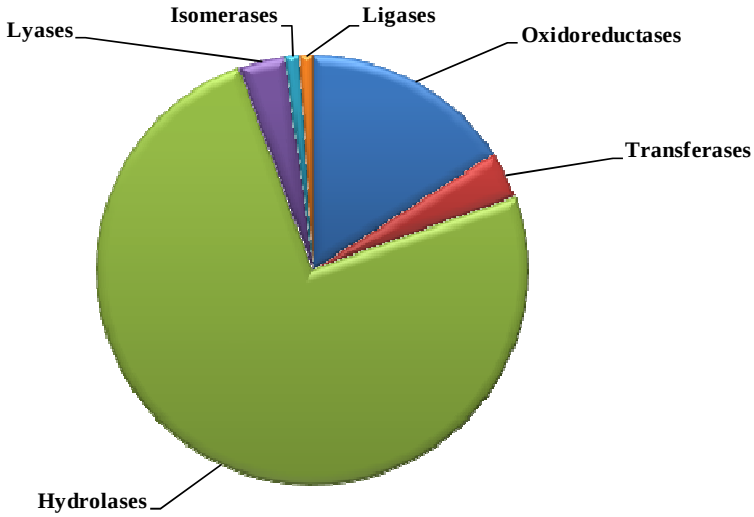


Figure 4. The relative use of enzyme classes in industry (FABER 2004)

As shown in Figure 4, more than 75% of industrial enzymes are hydrolases. Protein-degrading enzymes constitute about 40% of all enzyme sales. Proteinases have found new applications but their use in detergents is the major market. Lipases are one of the most important groups of biocatalysts for biotechnological applications. Based on forecasts the global enzyme demand (carbohydrases, proteases, polymerases and nucleases, lipases at present account for less than 5% of the market) are expected to rise 6.3 % annually through 2013, driven by strong demand in the special enzymes segment and good growth in animal feed and ethanol markets. It was nearly $5.1 billion in 2009 (*www.freedoniagroup.com/World-enzymes.html).*

As this work focuses on lipases which are hydrolases thus in the following paragraphs the characteristics of this enzyme group will be discussed in details.

2.3. Hydrolases

Hydrolases catalyze the addition of water to a substrate by means of a nucleophilic substitution reaction. Hydrolases are the most commonly used biocatalysts in organic synthesis. They have been used to produce intermediates for pharmaceuticals and pesticides. For modification of fats and oils, cocoa butter equivalent synthesis, for biofuel and for flavor enhancement. Among hydrolases there is particular interest towards amidases, proteases, esterases and lipases. These enzymes catalyze the hydrolysis and formation of ester and amide bonds. Lipases can hydrolyze

triglycerides into fatty acids and glycerol. They have been used extensively to produce optically active alcohols, acids, esters and lactones by kinetic resolution. The natural function of most hydrolases in vivo is to hydrolyze natural compounds (e.g., acid derivatives to free acids). In organic solvents it is possible to run such reactions in the reverse direction, allowing for synthesis of esters and amides. In most applications, biologically active compounds are needed in enatiomerically pure forms for use as drugs or agrochemicals. Therefore the ability to prepare enantiomerically pure compound has become a key issue in organic chemistry. Being proteins built from naturally occurring chiral, enantiopure amino acids, hydrolases are also enantiomerically pure, chiral polymers. Thus when reacting with a chiral, racemic substrate, they will react faster with one enantiomer of the substrate than the other. This enantioselectivity is the basis of the widespread use of hydrolytic enzymes and so lipases.

2.3.1. Lipases

Sources of lipases

Lipases [triacylglycerol acylhydrolases (EC 3.1.1.3)] are ubiquitous in nature and produced by various plants, animals and microorganisms. Lipases of microbial origin, mainly bacterial and fungal, represent the most widely used class of enzymes in biotechnological applications and organic chemistry (GUPTA et al. 2004).

Microbial enzymes are more useful than enzymes derived from plants and animals because of the great variety of catalytic activities available, the high possible yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable and safer than their corresponding plant and animal enzymes and their production is more convenient. Microbial lipases have already established their vast potential regarding their usage in numerous applications (WHITAKER et al. 2003, HASAN et al. 2006).

2.3.1.1. Structure

Lipases are serine hydrolases, which act at the lipid-water interface. Lipases possess an active site consisting of the catalytic triad composed of Ser-Asp/Glu-His that contains of the amino acids serine, aspartic or glutamic acid and histidine). Lipases usually contain a consensus sequence too, a highly conserved pentapeptide (Gly-X-Ser-X-Gly) forming a characteristic β - turn-α motif named the “nucleophilic elbow” is found around the active site serine. The three dimensional structures of lipases reveal the characteristic α/β–hydrolase fold.

The active site of lipases has a substrate-binding pocket and a hydrophobic tunnel, which may be responsible for the different substrate specificity and catalytic properties of this group of

enzymes. Also, these comparisons reveal two distinct regions in the hydrophobic tunnel: a phenylalanyl-rich region and an aliphatic-rich region. Whereas this last region is somewhat identical, the phenylalanyl content is specific for each lipase, resulting in a different environment of the catalytic triad residues, which probably fine tunes their lipase/esterase character (MANCHENO et al. 2003).

Lipases are characterized by the phenomenon called interfacial activation. A unique feature of all lipases is that the catalytic triad is buried under a “lid” (a helical oligopeptide) of a surface loop that must undergo a conformational change to open a channel for the active site for access of substrate such as a lipid droplet. The entrance to the active site is covered by this lid when dissolved in water. However, when in contact with an interface between water and apolar phase, the lid opens, allowing substrates to enter the active site. The interfacial activation of a lipase is caused by a change of its conformation at the interface of two immiscible phases (WONG 1995, VERGER 1997, TURNER et al. 2001, DIKS 2003, LOTTI and ALBERGHINA 2007).

The closed form, where the active site is isolated from the reaction medium by the lid, may be considered as inactive for many lipases. When in the reactive open-form the lid is displaced due to the interfacial activation, the active site is fully exposed to the reaction medium (VILLENEUVE et al. 2000, MATEO et al. 2007).

2.3.1.2. Mechanism

The mechanism involved in the action of lipases has been recognized to be very similar to the hydrolytic action of the serine proteases in many respects. Substrate hydrolysis starts with a nucleophilic attack on the carbonyl carbon atom of the ester bond, leading to the formation of the first tetrahedral intermediate stabilized by hydrogen bonding to nitrogen atoms of main-chain residues that belong to the so-called “oxyanion hole”. Hydrogen bonds between the negatively charged oxygen (oxyanion) of the tetrahedral intermediate and N-H peptide groups stabilize the negative charge on the oxyanion. This atomic and electronic environment has been called the oxyanionic hole. It stabilizes the tetrahedral structure and thus lowers energy required to activate the reaction. During the overall acido-basic catalysis, the carboxylic ester bond is broken and the alcohol group is released, taking with it one proton from the imidazolium ion of the histidine residue. The acyl chain of the ester bond remains covalently bound to the enzyme in the form of acyl-enzyme intermediate. This intermediate is in turn attacked by a water molecule, resulting in a second tetrahedral intermediate. The latter disintegrates, releasing the removed acyl group and thus the lipase is regenerated back into its initial state (Figure 5) (MALCATA 1996, (JAEGER and REETZ 1998, GUPTA et al. 2004, THONGEKKAEW and BOONCHIRD 2007).

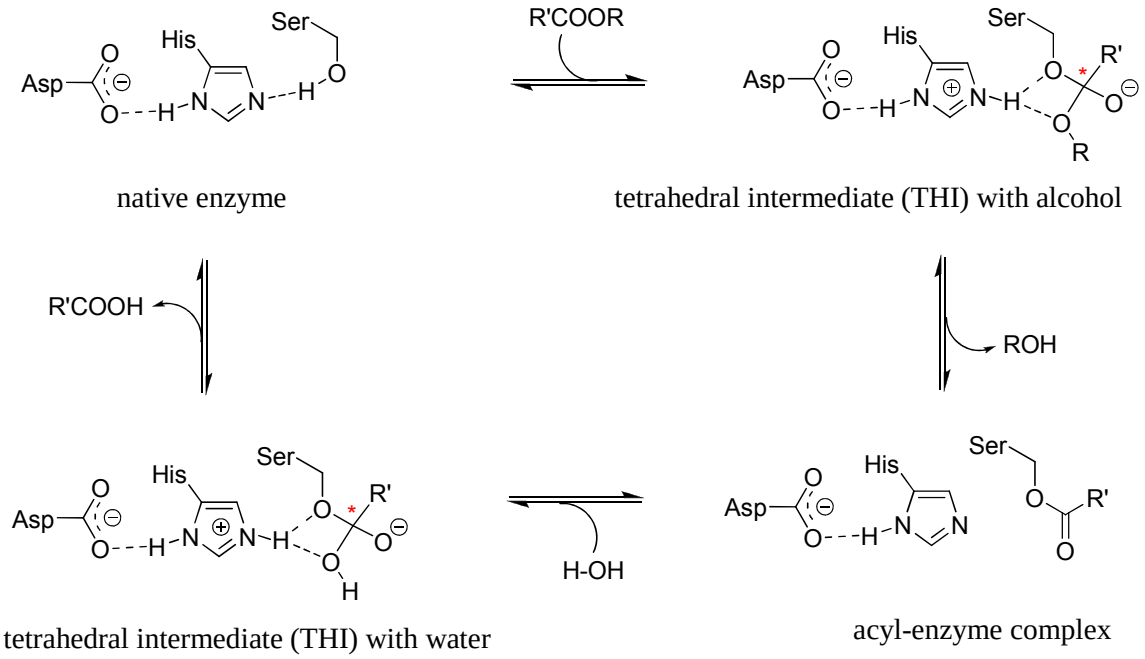


Figure 5. Operational mechanism of lipases, tetrahedral intermediate formation of lipases (WARSHEL et al. 1986 and 1989).

This mechanism is also known as *Ping Pong Bi Bi* mechanism (PAIVA 2000, REETZ 2002, DIKS 2003, LESKOVAC 2004).

It is a kinetic mechanism (Ping Pong Bi Bi) of lipase-catalyzed reactions involving multiple substrates.

A bisubstrate mechanism, in which the first product (P) is released before the second substrate (B) is bound, is called Ping Pong. Irrespective of the type of reaction catalyzes (i.e. hydrolysis, esterification, or interesterification), the most general, accurate, and accepted description of the catalytic action of lipases is a Ping Pong Bi Bi mechanism constituted by two major steps: (1) nucleophilic attack on the substrate ester bond by the oxygen atom of the hydroxyl group of Ser at the active site after opening the lid (thus resulting in formation of an acylated enzyme complex and release of the alcohol moiety of the original substrate and (2) hydrolysis of the acylated enzyme complex (thus resulting in formation of the product and regeneration of the enzyme).

A special multisubstrate reaction in which, for a two-substrate, two-product (i.e., bi-bi) system, an enzyme reacts with one substrate to form a product and a modified enzyme, the latter then reacting with a second substrate to form a second, final product, and regenerating the original enzyme.

Factors influencing lipase activity

Lipid hydrolysis depends on different parameters such as pH, temperature, water content, and the phase boundary area. The pH optimum of most of the lipases lie between pH 7.5 and 9.

Different microbial lipases are active between pH 5.5 and 8.5. Salts in different concentrations can also influence lipase activity.

2.3.1.3. “Lock and key” and induced fit model

"Lock and key" model

Enzymes are very specific, and it was suggested by Emil Fischer in 1894 that this was because both the enzyme and the substrate possess specific complementary geometric shapes that fit exactly into one another. This is often referred to as "the lock and key" model. However, while this model explains enzyme specificity, it fails to explain the stabilization of the transition state that enzymes achieve. The "lock and key" model has proven inaccurate and the induced fit model is the most currently accepted enzyme-substrate-coenzyme figure.

Induced fit model

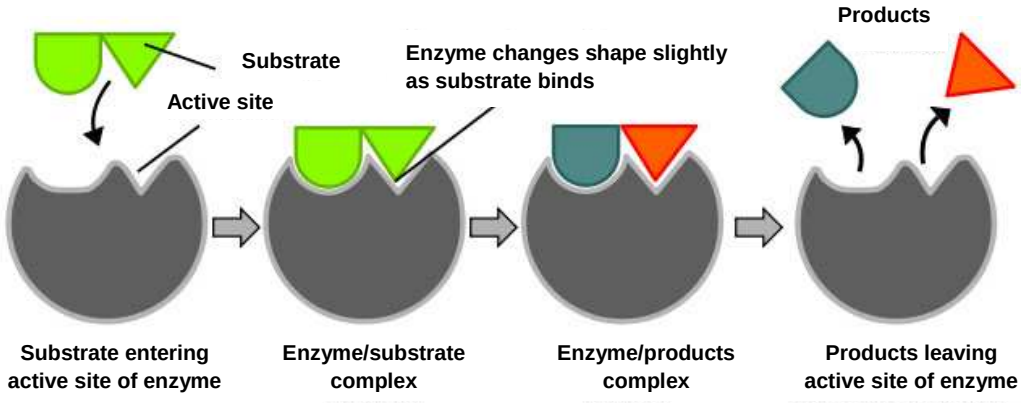


Figure 6. Induced fit hypothesis of enzyme action (www.websters-online-dictionary.org)

In 1958 Daniel Koshland suggested a modification of the lock and key model: since enzymes are rather flexible structures, the active site is continually reshaped by interactions with the substrate as the substrate interacts with the enzyme. As a result, the substrate does not simply bind to a rigid active site; the amino acid side chains which make up the active site are molded into the precise positions that enable the enzyme to perform its catalytic function. In some cases, such as glycosidases, the substrate molecule also changes their shape slightly as it enters the active site (VASELLA et al. 2002). The active site continues to change until the substrate is completely bound, at which point the final shape and charge is determined.

2.3.1.4. Typical reactions and applications

Lipases are hydrolases, which act under aqueous conditions on the carboxyl ester bonds present in triacylglycerols to liberate fatty acids and glycerol. The natural substrates of lipases are long chain triacylglycerols, which have very low solubility in water and the reaction is catalyzed at

the lipid-water interface. They are widely used as versatile biocatalysts in industrial biotechnology and modern organic chemistry. Under micro-aqueous conditions, lipases possess the unique ability to carry out the reverse reaction, leading to esterification, alcoholysis and acidolysis. Lipases have a very diverse substrate range, although they are highly specific as chemo-, regio- and enantioselective catalysts and catalyze wide range of enantio- and regioselective reactions such as hydrolysis, esterifications, transesterifications, and aminolysis (VAIDYA et al. 2008, LILJEBLAD et al. 2010).

The catalytic potential of lipases can be further enhanced and made selective by the novel phenomena of molecular imprinting and solvent engineering and by molecular approaches like protein engineering and directed evolution (GUPTA et al. 2004).

Synthetic application of novel biocatalytic methods is a continuously growing area of chemistry, microbiology and genetic engineering, due to the fact that biocatalysts are selective, easy-to-handle and environmentally friendly (POPPE and NOVÁK 1992, REHM et al. 1998, FABER 2004).

The broad substrate tolerance and unique catalytic performance of lipases have attracted growing interest throughout the world. As a consequence of their versatility in various applications, lipases regarded as enzymes of high commercial potential, especially those that are obtained from microbial sources. Lipases catalyze the hydrolysis of triglycerides at the oil/water interface, but their ability to form ester bound under reverse hydrolytic conditions enables them to catalyze various other types of reactions such as esterification, transesterification. Furthermore, lipases are enantioselective catalysts and can be used for the resolution of chiral compounds and the synthesis of a wide range of natural products, high-value pharmaceutical intermediates, fine chemicals, food ingredients and bio-lubricants (REETZ 2002, DIKS 2003, LOTTI and ALBERGHINA 2007, HE et al. 2010).

2.4. Functional foods

Fats and oils play an important role in our diet since thousands of years. They not only provides a concentrated source of energy for the body but from physiological point of view also certain components of fats are essential parts of our body cells and are needed to make hormones. Fat also helps to insulate our body and small amounts around the major organs have a protective effect. Several vitamins (vitamins A, D, E and K) are also fat-soluble and tend to be found in foods with a high fat content. Furthermore it contains essential fatty acids, carotenoids, plant sterols, phospholipids and natural antioxidants as well.

By the 21st century the (structure of) nutrition has changed significantly. At the end of 20th century obesity associated with lifestyle-related diseases gathered ground and became a serious

problem as main risk factors for other diseases (e.g. high blood pressure and cholesterol level, cardiovascular disease, diabetes, colon cancer etc.). The relation among dietary intakes of fatty acids, blood cholesterol levels and risk for cardiovascular disease remains an important health issue.

According to the recommendations of nutritionists and dietetics no more than a third of calories can come from fats which should be equally divided between fats derived from animal source and oils derived from plants. Nowadays the consumption of saturated fats is high while based on latest studies the ratio of SFA: MUFA: PUFA should be 30:40:30 ideally for the human body and the optimal ratio of ω3: ω6 fatty acids should be 1:2 or 1:5 has been shifted towards 1:10-1:20. Thus the type of fat consumed is becoming more and more important than the amount of fat in our diet.

Consumers are becoming increasingly aware of importance of a healthier diet that includes beneficial fats as functional foods which contain bioactive components that reduce the risk of disease and/or promote good health. This led the food industry to be proactive in reformulation of their product toward healthier diet including beneficial fats which is very challenging. It appears that the rapid growth of market for functional and nutraceutical foods and consumer demand for healthier fats prompts industry to invest money in the use of lipases as "green" catalysts for the production of special lipids.

2.4.1. Structured and specific structured lipids

Structured lipids (SLs) refers to oils and fats containing polyunsaturated fatty acids (PUFA) (long chain, mainly essential fatty acids; $>C_{14}$) and medium (C_6-C_{12}) or short chain (C_1-C_4) fatty acids located randomly on the glycerol backbone. In specific structured lipids (SSLs), the different fatty acids are located uniformly at either the *sn*-2 or *sn*-1,3 positions of the glycerol. SLs and SSLs can be produced either by chemical or enzymatic modification of triacylglycerols and may have improved nutritional or functional properties.

There are several excellent reviews on enzymatic modification of oils and fats focusing on the production of SLs, SSLs or "value-added" triacylglycerols (XU 2000a, OSBORN and AKOH 2002, HAYES 2004, IWASAKI and YAMANE 2004, TRIVEDI and SINGH 2005, JACOBSEN et al. 2006, REIS et al. 2009).

Enzymatic interesterification or transesterification processes have many advantages. These both terms may refer to *a)* exchange between acyl moieties of two distinct triacylglycerols, *b)* exchange of acyl moieties of triacylglycerols with free fatty acids (acidolysis) or *c)* reaction of triacylglycerols with an alcohol (alcoholysis or glycerolysis) (CAMP et al. 1998, XU 2000b, HAYES 2004, AKOH and LAI 2005, OSORIO et al. 2008).

The enzymatic methods afford milder processing conditions and the possibility of regiospecificity and fatty acid specificity (YANKAH and AKOH 2000, AKOH and LAI 2005). Moreover, it does not alter the fatty acid composition or the unsaturation level of the starting mixture, (CAMP et al. 1998, YANKAH and AKOH 2000) and thus eliminates any health concerns associated with *trans*-fatty acids.

Triacylglycerols rich in medium-chain acyl groups have many applications, including clinical treatments for patients with lipid absorption or digestion disorders and high energy nutraceuticals for athletes (HAYES 2004, MU and PORSGAARD 2005). Medium-chain acyl groups are readily and rapidly metabolized *via* portal vein and generally not stored in adipose tissue or cells.

The nutritional value and physicochemical properties of triacylglycerols are determined not only by their fatty acid composition, but also on the positional distribution of the acyl groups bonded to glycerol (SOUMANOU et al. 1998). Since groups at the *sn*-2 position are more strongly absorbed *in vivo* than those at the *sn*-1 or 3 positions, the SSLs that include an essential fatty acid in the *sn*-2 position are valuable nutrition sources for patients with pancreatic deficiencies (OSBORN and AKOH 2002, ST-ONGE et al. 2003, BOURQUET et al. 2003, JACOBSEN et al. 2006). The MLM-type structured triglyceride which contains medium-chain fatty acids (M) in *sn*-1 and *sn*-3 positions and long unsaturated fatty acids (L) in the *sn*-2 position can provide rapid delivery of energy *via* oxidation of the more hydrophilic medium-chain fatty acids, while at the same time providing an adequate supply of essential fatty acids from the remaining *sn*-2 monoglyceride, (SOUMANOU et al. 1998, IWASAKI and YAMANE 2004).

2.4.2. Sterols and sterolesters

Phytosterols (PSs, plant sterols) are typically obtained from natural sources; most frequently from the processing of plant oils (CEREOL NÖVÉNYOLAJIPARI RT. 2003). The chemical structure of PSs and cholesterol are similar. Thus, PSs may displace cholesterol by competing with receptor and/or carrier sites in the cholesterol absorption process (MOREAU et al. 2002, AKOH and LAI 2005, VILLENEUVE et al. 2005, BUNGE OILS INC. 2005). PSs may help to decrease the cholesterol level in blood, serve as feed stocks for hormone drugs, increase the response time and effectiveness of white blood cells against cancer and enhance the overall dietary efficacy. Therefore, the use of PSs is suggested in oil-based compositions (MOREAU et al. 2002, ST-ONGE et al. 2003, SEKI et al. 2003, BUNGE OILS INC. 2007, RESHMA et al. 2008 and KIM et al. 2008). The oil-soluble and fatty acid esters of phytosterols (PSEs) possess the same capabilities as PSs. Due to their good solubility in oils and their GRAS (Generally Recognized as Safe), status for use in margarines, (MOREAU et al. 2002) PSEs are generally preferred in the food industry, (MOREAU et al. 2002 and RODRIGUES et al. 2007).

2.4.3. Combination of specific structured lipids and phytosterol esters

In recent decades, several inventions and studies have been related to SLs and PSEs but just a few have been dealing with SLs and PSEs together (MOREAU et al. 2002, KIM et al. 2008, HELLNER et al. 2010). SLs combined with PSEs in the so-called healthy oil compositions have the advantages of both components in a variety of applications for promoting health and nutrition enhancement (MOREAU et al. 2002). The final products were obtained by chemical interesterification of an edible oil (soybean oil or corn germ oil, cottonseed oil, canola oil, safflower oil, sunflower oil, peanut oil or olive oil) with a medium-chain triglyceride (MCT) resulting in random SLs which were then physically mixed with PSEs. The blends of functional oil with PSEs were claimed as beneficial compositions for edible oils having good clarity, physical properties and cardio-protective effects (MOREAU et al. 2002).

The components of the SLs/SSLs − PSEs compositions can be manufactured by enzymatic methods using sterol esterase (EC 3.1.1.13) and lipase (EC 3.1.1.3) activities. Both activities may be present in commercial lipase preparations such as lipase from porcine pancreas (PPL, Amano), *Pseudomonas* sp. (Lipase PS, Amano), *Chromobacterium viscosum* (Lipase LP) and *Candida rugosa* (Lipase AY, Amano) (KONTKANEN et al. 2004). On the other hand, lipase preparations from *Aspergillus* sp. (Lipase A, Resinase A) exhibited no sterol esterase activity (MILLER et al. 2004). A present study on solid-state fermentation (SSF) of *Aspergillus* species demonstrated the significant sterol esterase activities of SSF products (TŐKE et al. 2007).

2.4.4. Structured lipids on the market - Functional applications of structured lipids

A functional lipid (as it was discussed above) is similar to a conventional fat or oil that is consumed as a part of a normal diet, but is demonstrated to have physiologic benefits, i.e. beyond serving a nutritional function, it may reduce the risk of chronic disease. The functionality of the lipids in food products varies from one product to another. The variation in functionality demands specific types of lipid for particular applications (Table 3).

Table 3. Commercial structured lipids

Product name or type	Supplier(s)	Composition	Application
Plastic fats (with zero *trans*-FA)	Unilever, Bunge	interesterified SCFA,MCFA and LCFA	margarine, modified butter, shortenings
Appetizer shortening		blend of fat 85-95% and oil 5-15%	shortening
Good –Fry oil	Good-Fry International N.V.	high-oleic corn or sunflower	frying (snacks, crisps)
Bohenin	Stephan Co.	glycerol 1,3-dibehenate 2-oleate $C_{18:1}$, $C_{22:0,:}$	prevent and slow down boom and stickiness of chocolates
Caprenin	Procter&Gamble Co's	$C_{8:0}$, $C_{10:0,}$ $C_{22:0}$	cocoa butter substitute
Salatrim (Benefat)	Danisco Cultor Food Inc.	$C_{2:0}$, $C_{3:0,}$ $C_{16\text{-}22:0}$	baked goods, filling fats
ARASCO	Martek Bioscience Corporation	~40% arachidonic acid	infant formulas
DHASCO	Martek Bioscience Corporation	single cell oil ~40% DHA	infant formulas
Enova Oil	ADM and Kao Corporation	special blend of soybean and canola oils with ~80% DAG	cooking, frying, salad dressing, baking
Lorenzo's Oil	Lorenzo's parents (Michaela and Augusto Odone)	$C_{22:1}$, $C_{18:1,}$ MCT and PUFA(ω3)	genetic disorder X-linked adrenoleukodystrophy
Betapol	Loders Croklaan	$C_{12:0}$, $C_{16:0,}$ $C_{18:1}$	human milk fat replacer
Captex	ABITEC	$C_{8:0}$, $C_{10:0,}$ $C_{18:2}$ $C_{12:0}$ $C_{18:1}$,$C_{18:2}$	cosmetics, clinical foods, beverages
Impact	Novartis Nutrition	High $C_{12:0}$, High $C_{18:2,}$	medical
Neobee	Stephan Co.	$C_{8:0}$, $C_{10:0,}$ ω6, ω3	nutritional/medical beverages, snack bars
Olestra	Procter&Gamble Co's	sucrose polyester, 0 calorie	savory snack

2.5. Enzyme immobilization

Nature has designed enzymes to catalyze reactions under physiological conditions, at ambient temperature and pressure at neutral pH. In contrast preparative chemical syntheses require high concentrations of reactants, organic solvents, high temperature, and pH to shift reaction equilibrium. Thus practical application of enzymes as catalysts for organic synthesis is limited. Enzyme immobilization is a good tool to bridge the above mentioned problem and an effective strategy to develop robust biocatalysts for industrial applications.

Preparing effective, suitable biocatalysts is very important as the catalytic activity, selectivity, specificity and enzyme stability are the key factors affecting biocatalytic efficiency (DRAUZ and WALDMANN 2002, BORNSCHEUER 2003). Thus engineering biocatalysts from enzymes as biological entities to industrial reactors is very challenging. There are several techniques available to modify or improve enzyme preparations with desirable physical and catalytic properties. Microbiology, chemistry, genetic engineering, protein engineering, are those sciences developed a lot in recent years while the main focus has been on directed evolution (REETZ 2002, DRAUZ K 2002, DIKS 2003). Apart from new trends the older fashioned techniques such as immobilization

(within physical, chemical modification area) was improved a lot as well in creating properly designed robust biocatalysts. For industrial applications, immobilization of biomolecules or microorganisms became a key issue in development of biotechnology. The primary advantage of immobilization is to increase the stability of the enzyme, as well as convenience in separation and recovery, and reuse of the enzyme from the product mixture (RODGERS et al. 2006, MATEO et al. 2007, NOUREDDINI and GAO 2007, LEE et al. 2010). Thus, immobilization of enzymes has been an active research topic in enzyme technology to enhance their activity, thermal and operational stability, and reusability (CAO 2005b, SHELDON 2007).

The aim of this section is to describe in general terms the classes of enzyme immobilization with the main focus on the sol-gel entrapment as it plays an important role in this thesis.

2.5.1. Methods of immobilization

The roots of enzyme immobilization can be traced back to 1916 when Nelson and Griffin carried out the bioimmobilization of invertase onto charcoal and it was shown that invertase remained catalytically active.

The basic methods of enzyme immobilization can be categorized into a few different, broadly defined groups e.g. covalent bonding, adsorption, cross-linking, entrapment and encapsulation (a-d on Figure 7).

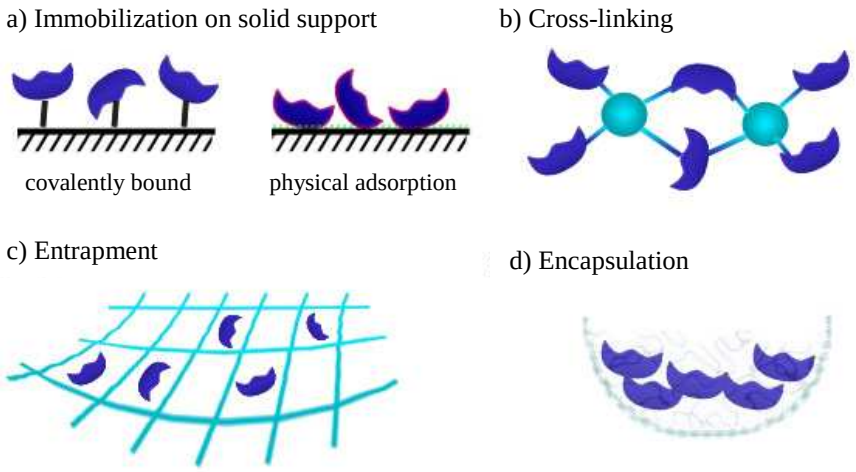


Figure 7. The basic categories of enzyme immobilization techniques (SEVELLA 2009)

Covalent enzyme immobilization:

Since 1950s' covalent enzyme immobilization has flourished and it is still an important method of enzyme immobilization, because covalent bonds usually provide the strongest linkages between enzyme and carrier.

Covalent bond is formed between the chemical groups of enzyme and chemical groups on surface of carrier. Covalent bonding is thus utilized under a broad range of pH, ionic strength and other variable conditions. Immobilization steps are attachment of coupling agent followed by an activation process, or attachment of a functional group and finally attachment of the enzyme.

Adsorption based immobilization:

An enzyme may be immobilized by bonding with low energy bonds (e.g. ionic interactions, hydrogen bonds, van der Waals forces, etc) to either external or internal surface of a carrier or support. Due to immobilization of enzymes on external surface, no pore diffusion limitations are encountered. These particles may have diameter ranging from 500 Å to about 1 mm.

Cross-linking

Cross-linking is characterized by covalent bonding between the various molecules of an enzyme via a polyfunctional reagent such as glutaraldehyde, diazonium salt, hexamethylene disocyanate, and N-N' ethylene bismaleimide. The fault of using polyfunctional reagents is that they can denature the enzyme. This technique is cheap and simple but not often used with pure proteins because it produces very little of immobilized enzyme that has very high intrinsic activity. It is widely used in commercial preparation.

Enzyme entrapment

Enzyme entrapment technique is one of the simplest methods for immobilization of enzymes and also for whole cells. Entrapment means that enzyme molecules or preparations are confined in a matrix formed by dispersing the catalytic component (a soluble/insoluble enzyme preparation) in a fluid medium (polymer solution), followed by formation of an insoluble matrix with confined enzymes by chemical or physical methods. So entrapment refers to the processes by which the enzymes are embedded in a matrix formed by chemical or physical means such as cross-linking or gelation. Matrix is generally formed during the immobilization process.

Enzyme encapsulation

Encapsulation means enclosing of a droplet of enzyme solution in a semipermeable membrane capsule. Encapsulation is the formation of a membrane-like physical barrier around an enzyme preparation. The method of encapsulation is cheap and simple but its effectiveness largely

depends on the stability of enzyme although the catalyst is very effectively retained within the capsule. This technique is restricted to medical sciences only.

A lot of variations based on the combinations of above mentioned methods, have been developed. The rational combinations of the available methods definitely facilitate the design of robust immobilized enzymes that can suit for various applications (CAO 2005a).

The possible advantages of immobilized enzyme process compared to the use of soluble enzyme are greater productivity per unit of enzyme, precise control of the reaction, continuous operation and automation of the process is possible, product does not contain the biocatalyst and the possibility to produce a unique product. Use of immobilized form of enzyme eliminated the need for downstream enzyme inactivation step that could be harmful for the product. (DIKS 2003, DRAUZ and WALDMANN 2002, HE et al. 2010).

There are some practical limitations of the usage of immobilized enzymes as well such as carrier cost, sometimes reduced activity of biocatalysts originated from steric hindrance and mass transfer limitations.

2.5.2. Kinetic behavior of immobilized enzyme systems

The catalytic behavior of enzymes in immobilized form may dramatically differ from the soluble enzymes. Mass transport effects (the transport of a substrate to the catalyst and diffusion of reaction products away from the catalyst matrix) may result in the reduction of the overall activity. Mass transport effects are usually divided into two categories external and internal effects. The external ones stem from the fact that substrate must be transported from the bulk solution to the surface of the immobilized enzyme. Internal limitations occur when a substrate penetrate inside the immobilized enzyme particle (Figure 8).

The effects of immobilization on the kinetic behavior of an enzyme can be classified as follows:

(1) Conformational and steric effects: the enzyme may be conformationally different when fixed on a support; alternatively it may be attached to the solid carrier in a way that would render certain parts of the enzyme molecule less accessible to substrate. These effects are the least well understood.

(2) Partitioning effects: the equilibrium substrate concentrations within the support may be different from those in the bulk solution. Such effects, related to the chemical nature of the support material, may arise from electrostatic or hydrophobic interactions between the matrix and low-molecular-weight species present in the medium, leading to a modified microenvironment, (i.e. different concentrations of substrate, product, hydrogen and hydroxyl ions) in the domain of the immobilized enzyme particle.

(3) Microenvironmental effects on the intrinsic catalytic parameters of the enzyme: such effects due to the perturbation of the catalytic pathway of the enzymatic reaction would reflect events arising from the fact that enzyme-substrate interactions occur in a different microenvironment when an enzyme is immobilized on a solid support.

(4) Diffusion or mass-transfer effects: such effects would arise from diffusion resistances to the translocation of substrate, product, or effect or from the site of the enzymatic reaction and would be particularly pronounced in the case of fast enzymatic reactions and configurations, where the particle size or membrane thickness is relatively large. An immobilized enzyme functioning under conditions of diffusion restrictions would be exposed, even in the steady state, to local concentrations of substrate and product different from those in the bulk (GOLDSTEIN 1976, SOARES et al. 2006, SEVELLA 2009).

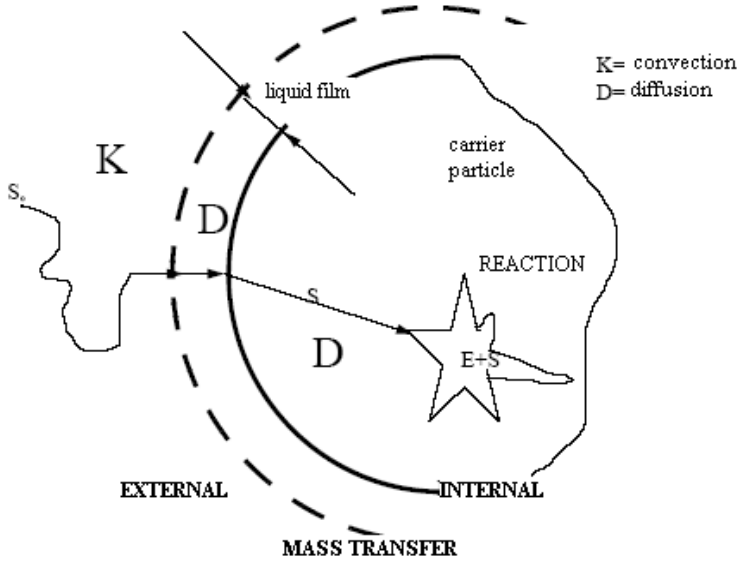


Figure 8. Mass transfer effects in carrier bound immobilized enzymes (SEVELLA 2009)

2.5.3. Sol-gel immobilization with and without carrier

Entrapment of enzymes in inorganic/organic hybrid polymer matrices has received a lot of attention in recent years (KIM et al. 2006, AVNIR et al. 2006). Sol-gel encapsulation has proven to be a particularly easy and effective way to immobilize purified enzymes, whole cells, antibodies and other proteins (HENCH and WEST 1990, AVNIR et al. 1994, ANVIR 1995). Furthermore this type of immobilization technique is favorable because it is not affected by microbial contaminations. The seminal works of Avnir (1994) and Reetz (2003) and their co-workers led to the generalization of this technique involving the acid-or base-catalyzed hydrolysis of tetraalkoxysilanes $Si(OR)_4$ (HENCH and WEST 1990) in the presence of an enzyme. Later studies indicated that variation of the silane precursors of e.g. different hydrophilicity enables alteration of

enzyme performance such as activity, stability or selectivity (REETZ et al. 2003, TOMIN et al. 2011). To increase retention of activity of enzymes it is usually necessary to use additives such as polyethylene glycol (PEG), crown-ethers or solid supports to modulate the pore size and increase the permeability of the substrate through the pores and the accessibility of the enzyme. (REETZ et al. 2003, RODGERS et al. 2006 and MATEO et al. 2007). A typical sol-gel immobilization process (Figure 9) involves acid- or base-catalyzed (Figures 10, 11) hydrolysis, then polycondensation of alkoxysilane precursor [$Si(OR)_4$] in the presence of additives to form a matrix in which the enzyme is encapsulated. The acid- or base-catalyzed (Figures 9, 10) hydrolysis can be resulted in different morphologies (Figure 12).

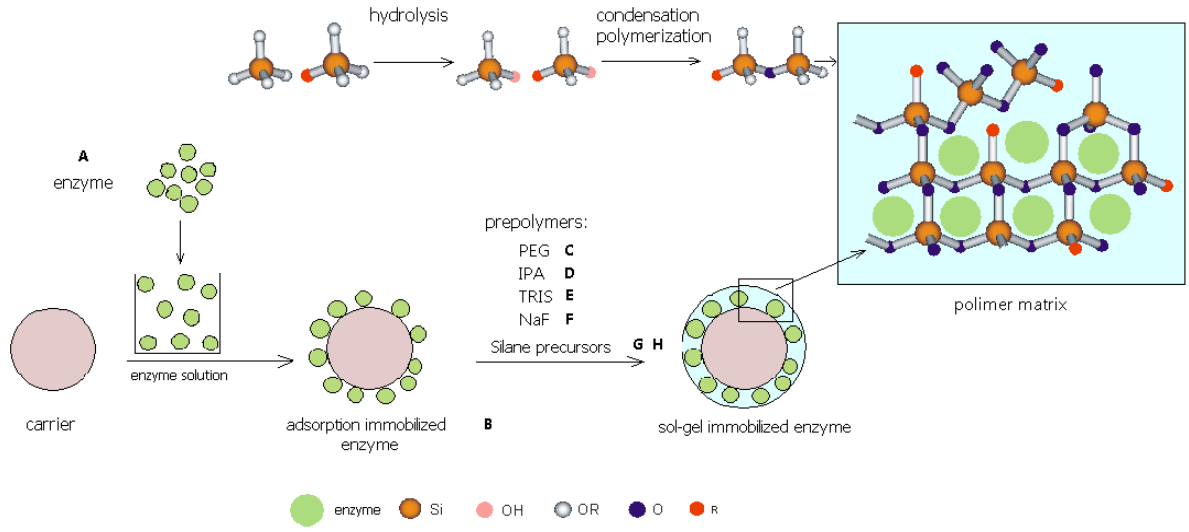


Figure 9. Process of sol-gel matrix development

Under acidic conditions, it is likely that an alkoxide group is protonated in a rapid first step. Electron density is withdrawn from the silicon atom, making it more electrophilic and thus more susceptible to attack from water.

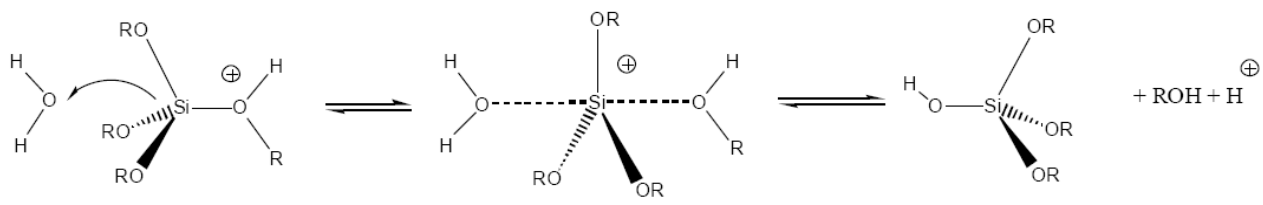


Figure 10. Acid-catalyzed hydrolysis

Base-catalyzed hydrolysis of silicon alkoxides proceeds much more slowly than acid-catalyzed hydrolysis at an equivalent catalyst concentration. Although hydrolysis in alkaline environments is slow, it still tends to be complete and irreversible.

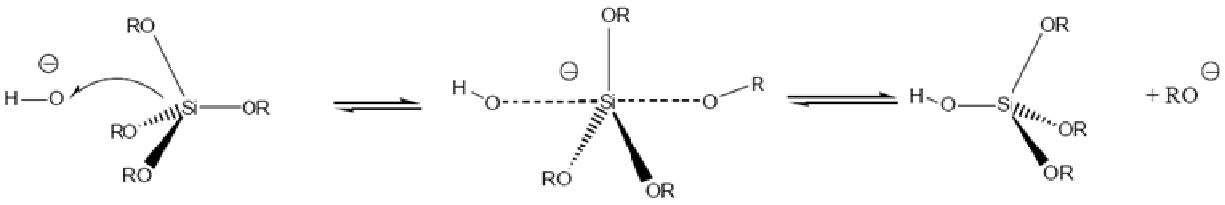


Figure 11. Base-Catalyzed Hydrolysis

Figure 12. Silica nanoscale network for silica gels derived from acid-catalyzed orbase-catalyzed hydrolysis and condensation. The acid-catalyzed morphology is more branched and polymeric while the base-catalyzed morphology comprises rough colloidal domains (Morris et al. 2001).

Using organically modified silanes of the type R'-Si$(OR)_3$, activity enhancement of the immobilized lipases could be observed with increasing amount and alkyl chain (R') length of the hydrophobic silanes (REETZ et al. 1995, KAWAKAMI and YOSHIDA 1996, REETZ et al. 1998, FURUKAWA and KAWAKAMI 1998, BORNSCHEUER 2003, REETZ et al. 2003, PÉTER et al. 2005, BRAUN et al. 2007).

The optimal molar ratios of the trialkoxy- and tetraalkoxysilane precursors [R'-Si$(OR)_3$ and Si$(OR)_4$] were also investigated (KAWAKAMI and YOSHIDA 1996, REETZ et al. 1998). The presence of additives like polyethylene glycol, polyhydroxy compounds or proteins (REETZ et al. 1996) can significantly enhance the catalytic activity of these enzymes. It was demonstrated that the presence of a small amount of isopropyl alcohol (IPA) during immobilization is beneficial (COLTON et al. 1995, CIPICIANI and BELLEZZA 2002). The characterization of the sol-gel biocatalysts is also well documented (NOUREDDINI and GAO 2007). In most cases, alkyltrimethoxysilanes (alkylTMOS's) were preferred for encapsulation of lipases. However, it was indicated that there is no significant difference in properties of encapsulated lipase biocatalysts prepared from alkylTMOS or alkylTEOS silane precursors (ZARCULA et al. 2009). Because alkylTEOS's are cheaper and the gelation time is longer and more controllable than with alkylTMOS's, the use of R'-Si$(OEt)_3$ and Si$(OEt)_4$ as silane precursors is preferable. The sol-gel immobilized lipases are supported on inert materials to reduce diffusion limitations of the substrates

or products to and from the enzyme and thus improve the reaction rate (WEHTJE et al. 1993, KAWAKAMI and YOSHIDA 1996, REETZ et al. 2003, VIDINHA et al. 2006, MEUNIER LEGGE 2010).

Although it was published that the nature of the R' substituent of the trialkoxysilane precursors may significantly influence the properties of the sol-gel lipases (REETZ et al. 1998 (FURUKAWA and KAWAKAMI 1998, BORNSCHEUER 2003, REETZ et al. 2003, PÉTER et al. 2005, BRAUN et al. 2007), only the binary sol-gel systems of trialkoxy- and tetraalkoxysilane precursors [R'-Si$(OR)_3$ and Si$(OR)_4$] for lipase immobilization have been studied. (REETZ et al. 1998, KAWAKAMI and YOSHIDA 1996). According to our best knowledge, ternary sol-gel systems of a tetraalkoxysilane and two different trialkoxysilane precursors [R^1-Si$(OR)_3$, R^2-Si$(OR)_3$ and Si$(OR)_4$] have been studied only for cutinase (EC 3.1.1.74) (VIDINHA et al. 2006).

Mesoporous silica materials can be also effective supports during immobilization (MICHAUX et al. 2010).

At the functional group level, two reactions are generally used to describe the sol-gel process: hydrolysis and condensation (alcohol and water). However, the characteristics and properties of a particular sol-gel inorganic network are related to a number of factors that affect the rate of hydrolysis and condensation reactions, such as, pH, temperature and time of reaction, reagent concentrations, catalyst nature and concentration, H_2O/Si molar ratio (R), aging temperature and time, and drying. Controlling these factors, it is possible to vary the structure and properties of the sol-gel-derived inorganic network over wide ranges.

2.5.4. Possible applications of sol-gel techniques

Sol-gel chemistry is a remarkably versatile approach for fabricating materials in a simple and cheap way. Scientists have used it to produce the world's lightest materials and some of its toughest ceramics. In sol-gel chemistry, nanometer-sized particles form and then connect with one another to create a three-dimensional (3D) solid network. Sol-gel materials can be produced in different forms, such as powders, films, fibers, and freestanding pieces of material called monoliths (Figure 13).

With sol-gel chemistry, it can be created a broad set of materials which can be applied as biocatalysts and highly specific sensors, for environmental, food and medical applications. Typical applications of sol–gel derived biomaterials include selective coatings for optical and electrochemical biosensors, stationary phases for affinity chromatography, immunoadsorbent and solid-phase extraction materials, controlled release agents, solid-phase biosynthesis and unique matrices for biophysical studies or applications such as optics coating, energy storage, ceramics, materials with controlled porosity, bioactive materials and nanoelectronics. (SOARES et al. 2006, TSENG et al. 2010).

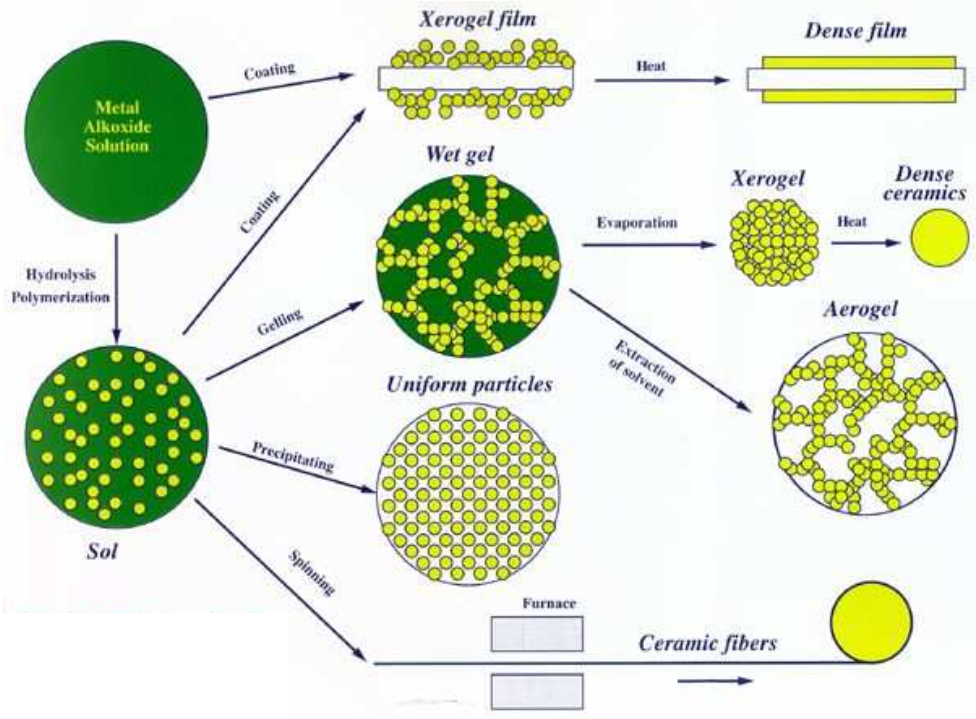


Figure 13. The sol-gel technologies and their products (www.chemat.com)

2.6. Bioimprinting

Enzyme immobilization technology has attracted increasing attention and considerable progress has been made in recent decades, because of progress in organic chemistry, protein chemistry and material science. This is still a rapidly growing field, because numerous new immobilization methods exist as the combination of more than two methods can be called unconventional methods. The combination of sol-gel immobilization and bioimprinting can be categorized as one member of unconventional methods' group.

The root of molecular imprinting is dated back to the experiments of Dickey in the 1940's who was inspired to create affinity for dye molecules in silica gel by a theory of Linus Pauling as to how antibodies are formed (1949). One of the most successful strategies for enhancing enzyme activity in organic solvents involves tuning the enzyme active site by molecular imprinting with substrates or their analogues (MINGARRO et al. 1995, GONZALES-NAVARRO and BARCO 1997, KAMIYA and GOTO 1998, RICH et al. 2002, FISCHMAN and COGAN 2003, CAO et al. 2009, YANG et al. 2010). Dual bioimprinting combining imprinting with protein surface coating and salt activation was reported recently (YAN et al. 2009). There are only a few publications on the mutual effects of sol-gel entrapment and bioimprinting influencing the activity and enantioselectivity of lipases (REETZ et al. 2003, CAO et al. 2009, HELLNER et al. 2011). Imprinting effects of ad hoc selected molecules involving lauric acid (LA), Tween 80 and other molecules (18-crown-6, methyl-β-cyclodextrin) were investigated so far (REETZ et al. 2003, CAO et al. 2009).

2.7. Kinetic resolutions with lipases

Conventional kinetic resolution procedures often provide an effective route for the preparation of enantiomerically enriched compounds. However, a resolution of two enantiomers will only provide a maximum of 50% yield of the enantiomerically pure material. This limitation can be overcome in a number of ways, including inversion of the stereochemistry of the unwanted enantiomer, racemization and recycling of the unwanted enantiomer or dynamic kinetic resolution.

The resolved starting material and product must be easily separable, preferably with minimal effort. Another feature of successful kinetic resolution is that starting material or product of high *ee* (>98%) is obtained near 50% conversion. Desirable characteristics of all catalytic asymmetric reactions, such as high yields, short reaction times, low catalyst loading, inexpensive catalysts, minimal generation of waste, reproducibility, broad substrate scope, functional group compatibility are all important considerations in kinetic resolutions. Kinetic resolutions can be performed on a preparative scale for the synthesis of large quantities of material (WHITAKER et al. 2003).

Kinetic resolutions are often the best option when racemate is inexpensive, when no reasonable method is available, or when classical resolution doesn't provide the desired material with high *ee* (DRAUZ and WALDMANN 2002, WALSH and KOZLOWSKI 2009).
Here I would like to refer to Section 2.2.7., where the selectivity was discussed in details.

2.7.1. Batch mode

The vast majority of the enzymatic enantioselective processes was performed in batch mode. Consequently, the main advantages of the flow-through approach − such as facile automation, reproducibility, safety, and process reliability − are not much exploited at research phase. Continuous-flow lipase-catalyzed kinetic resolution allows the rapid preparation of compounds with minimum workup (CSAJÁGI et al. 2008).

Esterification and transesterification reactions are commonly employed in industry using acids as catalysts at high temperature (100–300 °C) and pressure, which result in poor reaction selectivity, undesirable side products and low yields. The use of triacylglycerol lipases in chemical processes offers better quality of products, and the process can be more effective due to higher selectivity and fewer environmental problems (DOSSAT et al. 1999).

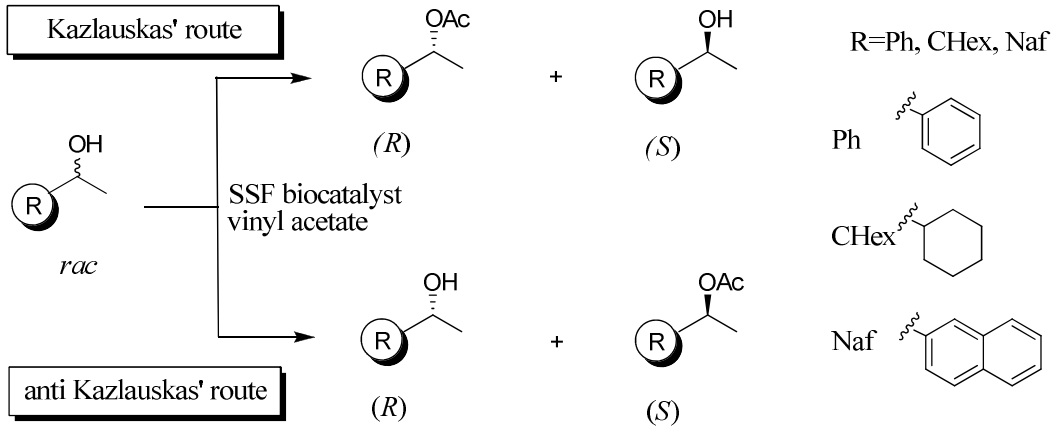


Figure 14. Kinetic resolutions of secondary alcohols by SSF biocatalysts

According to Kazlauskas rule in case of lipase-catalyzed kinetic resolutions of racemic secunder alcohols the (*R*)-enantiomer reacts/forms faster (Figure 14) (KAZLAUSKAS et al.1991).

Numerous lipase-catalyzed reactions in continuous packed-bed reactor systems were studied in connection with food and related industries. In this respect, mostly biotransformations of triglycerides and related compounds were performed in continuous-flow reactors. For example, lipase-catalyzed interesterification of butterfat with rapeseed oil (RØNNE et al. 2005) was investigated both in batch and in continuous reactions. Surprisingly, there are only a few examples for hydrolase-catalyzed enantioselective processes carried out in various continuous-flow systems (CSAJÁGI et al. 2008).

2.7.2. Continuous-flow system

As a prerequisite for any successful industrial process is rational design and development, taking into consideration both the technical and economic aspects. The goal of rational process development is to maximize selectivity, yield, chemical and enatiomeric purity as well as profitability, while trying to minimize catalyst, energy, raw materials and solvents consumption. Furthermore special attention has to be taken on characteristics of biocatalysts, biotransformation reaction and also on bioreactor in which this occurs.

Continuous processes could provide essential solutions to the problems (e.g. environmental, economic) occurs in batch mode reactions; however integrated flow technologies that could cover from lab scale to pilot and production scale exist only scattered today. There are only a few examples of hydrolase-catalyzed enantioselective processes carried out in continuous-flow systems. (PATEL et al. 1996, CHEN and TSAI 2000, UJANG et al. 2003).

Among others in our research group it was also found that stainless-steel continuous-flow packed-bed bioreactors could be effectively used to study the effects of temperature, pressure and flow rate on lipase-catalyzed kinetic resolution and provides a good tool to evaluate the

performance and efficiency of designed biocatalysts (CSAJÁGI et al. 2008 and TOMIN et al. 2010a).

In our research group the batch and continuous flow kinetic resolutions have already been investigated. For comparison of the continuous-flow and batch reactions, several of their features can be considered. One of the general feature of the kinetic resolutions is the well characterized degree of enantiomer selectivity (*E*) which – under irreversible reaction conditions [e.g. using vinyl esters in transesterifications (POPPE and NOVÁK 1992, FABER 2004)] – can be calculated from two of the following three properties: the conversion (*c*), the enantiomeric excess of the product (ee_P) and enantiomeric excess of the remaining substrate (ee_S) (CHEN et al. 1982, RAKELS et al. 1993). Thus, the value of *E* can be determined from *c* and ee_P, from *c* and ee_S (CHEN et al. 1982) or from ee_P and ee_S (RAKELS et al. 1993). In addition to their selectivities, the productivity of the reactions can also be characterized by the so called specific reaction rate (*r*) which indicates the amount of the product that can be formed in a minute by one g of enzyme. The specific reaction rate for a continuous-flow system (r_{flow}) can be calculated (TOMOTANI and VITOLO 2007) from the concentration of the product ([*P*] [μmol mL^{-1}]), the flow rate (*f* [mL min^{-1}]) and the mass of the applied enzyme (m_e [g]) according to Equation 7.

$$r_{flow} = \frac{[P] \times f}{m_e} \left[\frac{\mu\text{mol}}{\text{min} \times \text{g}} \right] \qquad \text{(Equation 7)}$$

A stirred (or shake flask) batch reaction can also be characterized by the specific reaction rate (r_{batch}) which can be calculated from the amount of the product (n_P [μmol]), the reaction time (*t* [min]) and the mass of the applied enzyme (m_e [g]) according to Equation 8 (CSAJÁGI et al. 2008).

$$r_{batch} = \frac{n_P}{t \times m_e} \left[\frac{\mu\text{mol}}{\text{min} \times \text{g}} \right] \qquad \text{(Equation 8)}$$

Although the above defined specific reaction rate can be calculated at any degree of conversion (*c*), rigorous comparisons between the productivity of a continuous-flow reaction and its batch mode counterpart using their *r* values can only be made at the same degree of conversions (as the rate of the product formation is not a linear function of *c*).

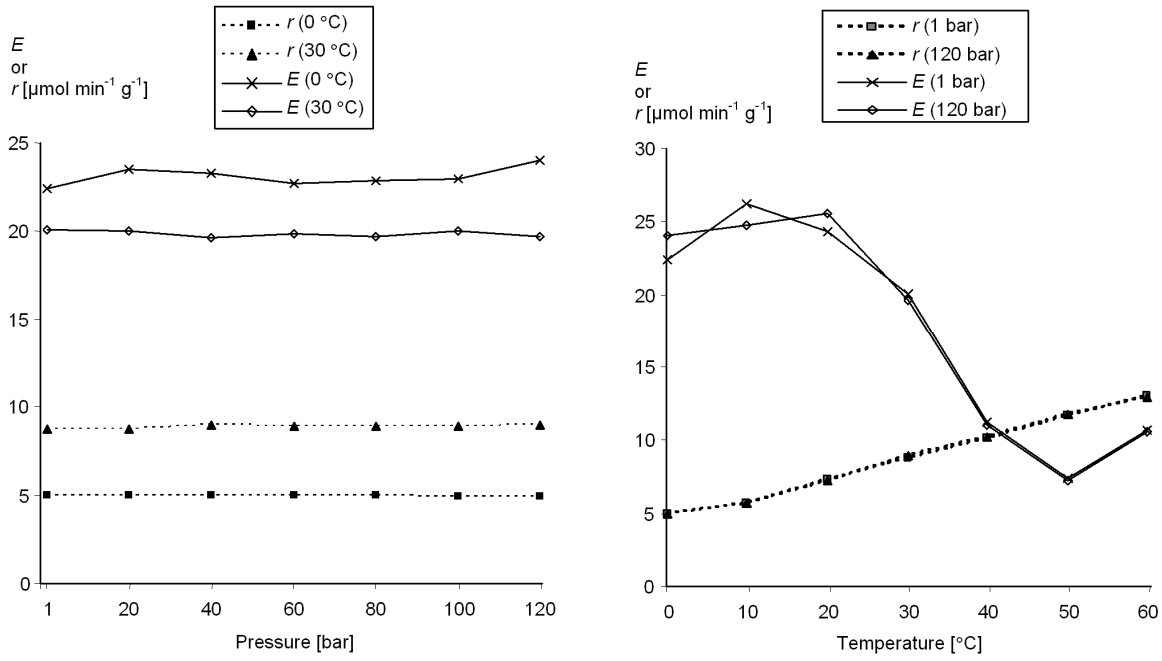


Figure 15. A) The effect of pressure and B) temperature on kinetic resolutions of *rac*-1-phenylpropan-2-ol in continuous flow reactor (CSAJÁGI et al. 2008)

In a previous study of our research group the effect of temperature (0-60 °C) and pressure (1-120 bar) on the continuous-flow mode kinetic resolution of racemic 1-phenylpropan-2-ol with vinyl acetate in a bioreactor filled with commercial immobilized lipase B from *Candida antarctica* indicated apparently no effect of the pressure on the selectivity (*E*) and productivity (*r*) of the reaction. As expected, productivity (*r*) increased monotonously with the temperature (Figure 15) (CSAJÁGI et al. 2008).

These results were found promising to develop immobilized biocatalysts and test them in continuous flow system.

3. OBJECTIVES

The objective of this research work is to provide biocatalytic methods for enzymatic one-pot synthesis of specific structured lipids and phytosterol esters and the systematic study of sol-gel immobilization method. Furthermore, a novel unconventional enzyme immobilization technique for lipase preparation as food and fine chemical application was presented.

The main objectives of this thesis can be summarized as follows:

- to develop integrated enzymatic methods starting from natural vegetable oil, a medium-chain fatty acid and phytosterol which result in a similar "value-added" blend of structured lipids and phytosterol esters that would be useful as a beneficial food additive. With special emphasis on using starting materials from natural sources and using solely enzymatic methods to avoid the increase of the unwanted *trans*-fatty acid content.
- to find the best process variant for the synthesis of structured lipids and phytosterol esters mixture
- to perform a systematic study of the sol-gel immobilization of a Celite-supported lipase using ternary silane precursor systems of various alkyltriethoxysilanes (alkylTEOS), phenyltriethoxysilane (PhTEOS) and tetraethoxysilane (TEOS).
- to reveal the effects of adsorption / entrapment in composite immobilization.
- to evaluate the effects of binary and ternary precursor systems in composite sol-gel immobilization.
- to investigate the imprinting effect of rationally selected molecules (substrate mimics in experimental X-ray structures of lipases and their structural analogues) in sol-gel process.
- to evaluate the biocatalytic efficiency of the developed enzymes using multisubstrate mixtures in continuous-flow reactor.
- to develop robust immobilized biocatalysts for continuous-flow kinetic resolutions.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Biocatalysts

Novozym 435 (lipase B from *Candida antarctica* – recently named as *Pseudozyma antarctica* – immobilized on acrylic resin; non-specific), lipase from Candida sp. *recombinant*, expressed in *Aspergillus niger* (Novozym CaLB L), Novozym carrier and Lipozyme TL IM (lipase from *Thermomyces lanuginosus* immobilized on silica; 1,3-specific) were donated by Novozymes A/S (Bagsværd, Denmark). CaLA-T2-150 (lipase A from *Candida antarctica* (CaLA, registered trademark from Novozymes Bagsværd, Denmark) covalently attached to dry acrylic beads) was the product of Chiral Vision BV.

Amano Lipase PS (lipase from *Burkholderia cepacia (*syn.: *Pseudomonas cepacia)*; >30.000 U/g; non-specific) and Amano Lipase AK (lipase from *Pseudomonas fluorescens*; >20.000 U/g; non-specific) were donated by Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). Lipase A from *Candida antarctica* – recently named *Pseudozyma antarctica* – (Cat. No. 62287; 2 U/mg; 2-specific) and lipase from *Candida cylindracea* – recently named *C. rugosa* – (Cat. No. 62316, 2 U/mg; non-specific) were products of Fluka. Novozym® 435 (Lipase acrylic resin from *Candida antarctica*, recombinant, expressed in Aspergillus niger), Lipozyme, immobilized lipase from *Mucor miehei* – recently named as *Rhizomucor miehei* – Cat. No. 62350; 30 U/g; 1,3-specific) was purchased from Sigma.

The biocatalysts with sterol esterase activity in this study were the air-dried mass of solid state fermentation (SSF) of *Aspergillus oryzae* NRRL 6270 (*Ao*SSF) and *Aspergillus sojae* NRRL 6271 (*As*SSF) (TŐKE et al. 2007).

The sterol esterase preparations in this study were obtained by solid state fermentation (SSF) of *Aspergillus oryzae* (NRRL 6270) and *Aspergillus sojae* (NRRL 6271) (TŐKE et al. 2007). *Aspergillus oryzae* NRRL 6270 and *Aspergillus sojae* NRRL 6271 strains were obtained from the Northern Regional Research Centre, USDA, Peoria, Illinois, USA. They are safe (GRAS) cultures applied in the food industry in Far East countries for a long time (e.g., koji, soy sauce) (JORGENSEN 2007). The SSF was carried out on a solid wheat bran medium containing olive oil as inductor and wetted with a salt solution (TŐKE et al. 2007). The air dried SSF preparations of *Aspergillus oryzae* NRRL 6270 (*Ao*SSF) and *Aspergillus sojae* NRRL 6271 (*As*SSF) were used without any further purification.

4.1.2. Chemicals, solvents, raw materials

Caprylic acid was obtained from Sigma. The sunflower oil (48.3-74.0% linoleic acid and 14.0-39.4% oleic content) the starting phytosterols (CEREOL NÖVÉNYOLAJIPARI RT. 2003) and olive oil were the products of Bunge (formerly Cereol).

Caprylic acid and other compounds applied as GC and HPLC standards (oleic acid, 1,3-diolein, tricaprylin, triolein, trilinolein, 1,2-dilinoleoyl-3-palmitoyl-*rac*-glycerol, 1,2-dioleoyl-3-linoleoyl-*rac*-glycerol, 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol) were obtained from Sigma. β-Sitosterol (No. CNBI567152) and stigmasterol (No. 85860) used as GC standards were products of Merck and Fluka, respectively.

Long chain fatty acids (LCFA) were produced by hydrolysis of sunflower oil (GUBANOV et al. 2005, ARCHER-DANIELS-MIDLAND Company 2006). Caprylic acid methyl ester was synthesized according to the literature (GARTENMEISTER 1886). Long chain fatty acid methyl ester (LCFA Me) was prepared from LCFA and methanol analogously. 1,2-*O*-Isopropylidene glycerol was synthesized by known procedure (YU 2003).

The phytosterol (PS) composition was the product of Bunge (CEREOL NÖVÉNYOLAJIPARI RT. 2003). The composition of PSs from a mix of sunflower oil and rapeseed oil was checked by GC using commercial standards [brassicasterol (21.0 %, at 22.59 min), campesterol (26.7 %, at 23.30 min), stigmasterol (3.4%, at 23.57 min), β-sitosterol (48.9%, at 24.42 min) were identified as main phytosterol components].

2-propanol (IPA), and Geduran® Si 60 (for column chromatography) were products of Merck Chemicals.

Tris(hydroxymethyl)aminomethane and triethylamine were purchased from Reanal.

Vinyl acetate and sodium fluoride (NaF), sodium borohydride ($NaBH_4$), acetic anhydride, oleic acid (OA), polyethylene glycol 400 (PEG 400), Triton X-100 (polyethylene glycol *tert*-octylphenyl ether, TRX100), BRIJ 30 (polyethylene glycol dodecyl ether, BRIJ), tetraethylene glycol (TEG), hexan-2-ol (*rac*-**1c**), octan-2-ol (*rac*-**1d**), nonan-2-ol (*rac*-**1e**), decan-2-ol (*rac*-**1f**) and dodecan-2-ol (*rac*-**1g**), lauryl chloride were products of Sigma-Aldrich. 1-Phenylethanol (*rac*-**1a**), heptan-2-ol (*rac*-**1b**), polyethylenglycol 1000 (PEG), Celite® 545 (Cat. No 22140), tetraethoxysilane (TEOS), n-propyltriethoxysilane (PrTEOS) and phenyltriethoxysilane (PhTEOS), lauric acid (LA) 1-(thiophen-2-yl)ethanone were obtained from Fluka. n-Hexyltriethoxysilane (HexTEOS), n-octyltriethoxysilane (OcTEOS), n-decyltriethoxysilane (DecTEOS), n-dodecyltriethoxysilane (DodTEOS), n-octadecyltriethoxysilane (OctdTEOS), 1*H*,1*H*,2*H*,2*H*-perfluorooctyltriethoxysilane (PFOctTEOS) and dimethyldiethoxysilane (DMDEOS) were obtained from Alfa Aesar.

Tetraethylene glycol dilauryl ester (L-TEG-L) and 1-(thiophen-2-yl)ethanol (*rac*-**1h)** were synthesized in our laboratory.

Solvents (n-hexane, acetone, dichloromethane, methanol) obtained from Molar Chemicals were freshly distilled before usage.

4.2. Applied analytical methods

4.2.1. Analytical methods for structured lipids synthesis

The conversion of the phytosterol acylation and triacylglycerol transesterification reactions were checked on a Hewlett Packard 6890 GC equipped with FID detector, on-column injector and HP-1 column (25 m × 200 μm i.d. × 0.11 μm film thickness, Agilent Technologies) and a non-polar fused silica precolumn (0.53 mm i.d., Supelco) using H_2 as carrier gas at a flow of 3.0 ml/min. Oven temperature was programmed as follows: 35°C for 2 min, 35-50 °C at 4 °C/min, 50-200°C at 20 °C/min, 200-320°C at 5°C/min, 320-350°C at 4°C/min, and holding for 10 min. The detector was operated at 350 °C, while the injector was in track oven mode because of the on-column injection. ChemStation software was used to evaluate the chromatograms. Similarly as it was found earlier with the sterol esterase preparations obtained by solid state fermentation (SSF) of *Aspergillus* strains [Figure 1 of Ref. (TŐKE et al. 2007)], no significant variation has been found in the degree of esterification (conversion to sterol esters) for the different phytosterols. The main phytosterol components at >90 % conversion in acylation reaction of PSs with *Ao*SSF using caprylic acid were present between 31.1-32.8min [brassicasteryl caprylate (22.6%, at 31.18min), campesteryl caprylate (25.9 %, at 31.84 min), stigmasteryl caprylate (3.2 %, at 32.07 min), β-sitosteryl caprylate (47.6%, at 32.74 min)]. The main phytosterol components (PS esters with C_{18} acids) at >90% conversion in acylation reaction of PSs with *Ao*SSF using free fatty acids from sunflower oil were present between 40.5-41.4 min. No significant peaks from transesterification of sunflower oil with caprylic acid were present in these two regions.

The purity of the synthetic standards and regioisomeric composition of the triglycerides in the final reaction products were analyzed on Alliance Waters 2690 HPLC equipped with an evaporative light scattering detector (PL-ELS 1000, Polymer Laboratories, operating at 40 °C) and an Ultra C-18 column (250 x 4.6 5μm, Restek) using a binary solvent gradient (solvent A: acetonitrile and solvent B: dichloromethane; 0-25 min: 30% B, 25 51% B, 26 70% B, 27 70%B, 28 30% B. The total run time was 30 minutes) at a flow rate of 0.72 ml/min. Each sample was diluted in 70/30 A/B solution and was stored at 4°C. Peak identification was based on comparison with purchased and prepared standard triglycerides and on equivalent carbon number (ECN) method (BERGQVIST KAUFMANN 1993, BUCHGRABER et al. 2004 and OSORIO et al. 2008). The ECN number of MLM, MML structured lipids are 30-32 and for LML, LLM are 36-40.

The NMR spectrum was recorded in $CDCl_3$ on a Bruker DRX-500 spectrometer (at 500 MHz for ^{1}H- and 125 MHz for ^{13}C-spectra).

4.2.2. Standards for analysis of the reaction products

4.2.2.1. Tricaprylin

Tricaprylin was synthesized from caprylic acid and glycerol using DCC (LIE KEN JIE and LAM 1995).

4.2.2.2. 2-Capryloyl-1,3-dioleoyl-gylcerol (LML*)*

1,3-Diolein standard (100 mg, 0.16 mmol) and caprylic acid (30 mg, 0.21 mmol) were stirred at 60°C, under vacuum (30-40 mmHg) for 15 minutes. Then DCC (33 mg, 0.16 mmol) and DMAP (5 mg) were added and the resulting mixture was stirred at 60 °C under vacuum (10-15 mmHg) for 1.5 h. After cooling, diethyl ether (10 ml) and hexane (10 ml) were added to the reaction mixture and the solid residue was removed by filtration. The filtrate was washed with 15 % HCl (2 × 10 ml), saturated $NaHCO_3$ (3 × 10 ml) and brine (2 × 10 ml). After drying the organic layer on anhydrous Na_2SO_4, the solvents were removed by vacuum rotary evaporator to give the LML triglyceride (73 mg, 61 %) as oil. The purity of LML was checked by HPLC (curve A in Annex 1).

4.2.2.3. Synthesis of triglyceride standard containing capryloyl residue at *sn*-1 and *sn*-3 positions (MLM)

MLM was synthesized in a two-step chemo-enzymatic reaction using a modified method of enzymatic acylation of glycerol with caprylic acid (ROSU et al 1999).

To a mixture of glycerol (1.4 g, 15.2 mmol) and caprylic acid 5 g (35 mmol) *Candida antarctica* lipase B (Novozym 435, 800 mg) was added and the resulting mixture was stirred under vacuum (10-15 mmHg) at 60 °C for 1.5 h when TLC analysis indicated the presence of diglyceride (1,3-dicaprylin) almost exclusively. After removing the enzyme by filtration, the solvent was distilled off from the filtrate by rotary evaporation. Column chromatography of the residue (silica gel, hexane: acetone 10:0.5 to 10:1 v/v%) resulted in 1,3-dicaprylin (2.1 g, 40 %) as semisolid at room temperature. The product was uniform by HPLC and its ^{1}H and ^{13}C NMR spectra agreed with those reported (HALLDORSSON et al. 2003).

In the second step, the purified 1,3-dicaprylin (0.5 g, 1.45 mmol) was added to a mixture of DCC (0.3 g, 1.45 mmol), catalytic amount of DMAP (10 mg) and long chain fatty acids (LCFA) (0.53 g, 1.89 mmol). The resulting mixture was stirred at 60 °C under vacuum (10-15 mmHg) for 1 h. After cooling the reaction to room temperature, diethyl ether (50 ml) and hexane (50 ml) were added to the reaction mixture and the solid residue was removed by filtration. The filtrate was washed with 15 % HCl (2 × 40 ml), saturated $NaHCO_3$ (3 × 20 ml) and brine (2 × 20 ml). After

drying the organic layer on anhydrous Na_2SO_4, the solvents were removed by vacuum rotary evaporator. Careful chromatography of the residue (silica gel, hexane: acetone 5:0.2 to 10:1 v/v%) gave MLM (78 mg) as an oil. The MLM standard was characterized by HPLC (curve B in Annex 1).

4.2.2.4. Synthesis of triglyceride standard containing capryloyl residue at sn-1 / sn-3 position (LLM)

The LLM triglyceride sample was obtained by a three-step synthesis from 1,2-*O*-isopropylidene glycerol, caprylic acid and long chain fatty acids (LCFA) from sunflower oil by a three step process involving DCC coupling of LCFA to racemic *sn*-1 / *sn*-3-caprylic-glycerol as final step.

The caprylic ester of 1,2-*O*-isopropylidene glycerol was prepared analogously as described for the corresponding stearic ester (YU 2003) by stirring a mixture of methyl caprylate (0.73 g, 5 mmol) and 1,2-*O*-isopropylidene glycerol (1.01 g, 7.6 mmol) and Na_2CO_3 (17.6 mg, 0.16 mmol) in a flask equipped with air cooler using an oil bath at 140 °C. After the reaction was completed (6 h), the excess 1,2-*O*-isopropylidene glycerol was removed by vacuum evaporation (10 mmHg). After adding diethyl ether (50 ml) to the residue, and the resulting mixture was washed with water (2×10 ml) and dried over $MgSO_4$. The solvent was removed in vacuum and the residue was purified by chromatography (silica gel, hexane-acetone 5:2) to give (2,2-dimethyl-[1,3]dioxolan-4-yl)methyl caproate (455 mg) as an oil. The ^{1}H and ^{13}C NMR spectra of the product agreed with literature data (BATOVSKA et al. 2004).

A mixture of the resulting (2,2-dimethyl-[1,3]dioxolan-4-yl)methyl caproate (0.403 g, 1.5 mmol), 95 %, ethanol (3.0 ml) and Amberlyst 15 (90 mg) was stirred under reflux for 4.5 h. After filtration the filtrate was concentrated in vacuum and the residue was purified by column chromatography (silica gel, hexane: acetone 10:0.5 to 1:1) to give 1-capryloyl glycerol (58 mg). The ^{1}H and ^{13}C NMR spectra of the product agreed with literature data (BATOVSKA et al. 2004).

After stirring a mixture of 1-capryloyl glycerol (50 mg, 0.23 mmol), LCFA (0.16 g, ca. 0.57 mmol) at 60°C under vacuum (30-40 mmHg) for 15 min, DCC (47 mg, 0.23 mmol) and DMAP (10 mg) were added and the resulting mixture was stirred at 60 °C under vacuum (10-15 mmHg) for 3.5 h. After cooling the reaction to room temperature, the product was purified by a similar procedure as described for MLM (Section 2.2.3.) to yield LLM (15.4 mg) as oil. The LLM standard was characterized by HPLC (curve C in Annex 1).

4.2.2.5. Synthesis of triglyceride standard containing capryloyl residue at *sn*-1/*sn*-2 or *sn*-2/*sn*-3 positions (MML)

The MML triglyceride standard was obtained by a three-step synthesis from 1,2-*O*-isopropylidene glycerol, caprylic acid and long chain fatty acids (LCFA) from sunflower oil by a three step process involving DCC coupling of caprylic acid to racemic *sn*-1 / *sn*-3-LCFA glycerol as final step.

The LCFA ester of 1,2-*O*-isopropylidene glycerol was prepared analogously as described for the corresponding stearic ester (YU C.C., 2003) by stirring a mixture of LCFA Me (0.9 g, 3.04 mmol) and 1,2-*O*-isopropylidene glycerol (0.6 g, 4.54 mmol) and Na_2CO_3 (10 mg) in a flask equipped with air cooler using an oil bath at 140 °C. After the reaction was completed (6 h), the reaction was purified as in the first step of the LLM process (Section 2.4) to give LCFA (mainly linoleate) ester of 1,2-*O*-isopropylidene glycerol (698 mg) as an oil.

A mixture of the resulting LCFA (mainly linoleate) ester of 1,2-*O*-isopropylidene glycerol (650 mg), 95 %, ethanol (2.5 ml) and Amberlyst 15 (70 mg) was stirred under reflux for 5 h. After filtration the filtrate was concentrated in vacuum and the residue was purified by column chromatography (silica gel, hexane: acetone 10:0,5 to 1:1) to give 1-LCFA glycerol (67 mg).

After stirring a mixture of 1-LCFA glycerol (67 mg, 0.19 mmol), caprylic acid (68 mg, 0.48 mmol) at 60°C under vacuum (30-40 mmHg) for 15 min, DCC (39 mg, 0.19 mmol) and DMAP (8 mg) were added and the resulting mixture was stirred at 60 °C under vacuum (10-15 mmHg) for 3.5 h. After cooling the reaction to room temperature, the product was purified by a similar procedure as described for MLM (Section 2.3.) to yield MML (12 mg) as oil. The MML standard was characterized by HPLC (curve D in Annex 1).

4.2.3. Scanning electron microscopy

The surface morphology of the samples was investigated with a JEOL JSM-5500LV scanning electron microscope. The samples of free lipase AK, lipase AK on solid support and supported lipase AK encapsulated in sol-gel matrices were coated with gold prior to analysis. Electron beam energy of 25 kV was used for the investigations.

4.3. Experimental

4.3.1. Screening of biocatalysts for production of specific structured lipids and phytosterol esters compositions

Biocatalyst screenings for production of structured lipids and phytosterol esters by acidolysis and esterification reactions were performed in shaken screw-capped test vials. Four different types

of reaction mixtures were investigated on 1 mmol scale (Type I-IV in Table 4, for details see footnote of Table 4). The Type I reactions consisted of phytosterol (414 mg, 1 mmol), sunflower oil and caprylic acid in a molar ratio of 1:2:12. The four types of reaction mixtures were shaken at ambient temperature or 50 °C at 1200 rpm. The progress of formation of structured lipids and phytosterol esters was monitored by TLC and GC by taking samples (50 µl, dissolved in 1 ml dichloromethane to stop the reaction) at 4, 24, 48, 72 h and at 6, 7, 8, 9, 10, 13 days. Seven commercially available lipase and two SSF sterol esterase preparations (TŐKE et al. 2007) were screened for production of structured lipids using Type II reactions (Table 5) and for synthesis of phytosterol esters using Type III reactions (Table 6) or Type IV reactions.

4.3.2. Enzymatic processes for one-pot production of specific structured lipids and phytosterol esters compositions

Two different biocatalysts, *Ao*SSF [preparation of *Aspergillus oryza*e NRRL 6270, for sterol ester formation (TŐKE et al. 2007)] and Lipozyme (immobilized lipase from *Mucor miehei*, for structured lipid formation), were used in the enzymatic processes. Six process variations with these biocatalysts (Types A-F in Table 7) were tested for integrated enzymatic one-pot processes for production of SSLs and PSEs on 1 mmolar scale (based on PSs) using standard reaction conditions with 0.12w/w% added water (based on the total mass of the starting compounds). Each reaction type was performed in three parallel series using three different compositions of phytosterol, sunflower oil and caprylic acid, in molar ratios of 1:2:12, 1:2:18 and 1:2:24. In each case, aliquots (50 µl, dissolved in 1 ml dichloromethane to stop the reaction) were taken daily and the progress of formation of structured lipids and phytosterol ester (Figure 18) was monitored by TLC, GC and HPLC (Figure 18).

For reaction type A, *Ao*SSF (400 mg) was added to the starting compounds in a closed screw-capped vial (10 ml) and the resulting mixture was incubated for three days (50°C, 1200 rpm). Then the forming water was removed from the opened vial in rotary evaporator (7 min at 30 mmHg using a heating bath of 50°C) and the mixture was incubated for 1 more day at 50°C and 1200 rpm. After addition of Lipozyme (100 mg), the mixture was incubated further (4 days, 50°C, 1200 rpm). Reaction type B was similar to type A except for the removal of the SSF sterol esterase from *A. oryza*e after the first four day period of incubation. Reaction type C was similar to type A, but without water removal at the 3rd day. Reaction type D was similar to type A, but worked up after the 4th day without addition of Lipozyme. For reaction type E, SSF sterol esterase from *A. oryza*e (400 mg) and Lipozyme (100 mg) were added to the starting compounds and the resulting mixture was incubated for four days (50°C, 1200 rpm). Reaction type F was similar to type E but with water removal at the 3rd day.

4.3.3. Development of the processing method for specific structured lipids and phytosterol esters mixtures

Removal of the excess caprylic acid from a mixture of structured lipids and phytosterol esters was modeled using a mixture (100 g) of caprylic acid (45%); oleic acid (8%); a blend of medium-chain triglyceride, rapeseed oil and sunflower oil (27%); and commercial phytosterol ester (20%). Caprylic acid was distilled off from this mixture under nitrogen atmosphere at 7-8 mbar and 120-130 °C resulting in yellow oil (54 g). SSLs and PSEs mixtures (~ 4 g crude mass) from reaction types A and B [starting from phytosterol (1 mmol), sunflower oil (2 mmol) and caprylic acid (12 mmol)] were treated similarly to yield the corresponding SSLs and PSEs mixtures as yellow oils (2.4 g for Type A and 2.2 g for Type B mixtures).

4.3.4. Sol-gel immobilization of lipase AK deposited on Celite® 545 using binary or ternary silane precursor systems

A two-step procedure was applied for sol-gel entrapment of Lipase AK on Celite 545.

Step 1: The lipase AK powder (50 mg for the standard procedure; 50, 125, 250, 375 or 500 mg for the enzyme loading tests) was added to TRIS-HCl buffer (0.1 M, pH 7.5, 780 μl) at 4°C with stirring for 10 minutes followed by addition of Celite 545 (500 mg). Acetone (10 ml) was added to the well stirred Celite-enzyme mixture at 10 ml min^{-1} rate at -18°C. The resulting solid was filtered off and left in air at room temperature for 12 hours for drying. The Celite-enzyme preparations were then used for sol-gel immobilization.

Step 2: TRIS-HCl buffer (0.1 M, pH 7.5, 390 μl), PEG solution (4% w/v, 200 μl), NaF solution (1M, 100 μl) and IPA (200 μl) were mixed in a 20 ml glass vial and the resulting solution was shaken at 1000 cycles per minute at room temperature for 10 minutes. During the continuous shaking, the corresponding silane precursors alkylTEOS-TEOS (1.5 mmol alkylTEOS and 1.5 mmol TEOS) or the mixture of alkylTEOS : PhTEOS : TEOS (3 mmol; the alkylTEOS : PhTEOS : TEOS molar ratio varied from 0.1 : 0.9 : 1 to 0.9 : 0.1 : 1 in 0.1 steps) and Celite-enzyme (250 mg) were added to the vial resulting in a sol suspension. To complete the polymerization, the mixture was shaken for 12 hours at room temperature. The formed solid was washed with IPA (7 ml), distilled water (5 ml), IPA (5 ml) and n-hexane (5 ml). The resulting white powder was dried in a vacuum exicator for 5 h (until 0.4 mmHg final level of vacuum). The sol-gel lipase AK preparations were stored at room temperature.

4.3.5. Activity and enantiomer selectivity tests of the Lipase AK preparations

The free or sol-gel immobilized Lipase AK (50 mg) was added to the solution of racemic test alcohol (1-phenylethanol, *rac*-**1a**, 49 mg, 0.4 mmol; or 2-heptanol, *rac*-**1b**, 46 mg, 0.4 mmol) and

vinyl acetate (100 µl) in hexane - THF 2:1 (1 ml), and the resulting mixture was shaken at 30°C in a sealed glass vial at 1000 cycles per minute. For GC analyses, samples were taken directly from the reaction mixture (sample size: 10 µl, diluted with CH_2Cl_2 to 100 µl) at 2, 4 and 8 hours. The esters were analyzed on an Acme 6100 instrument (Young Lin Instrument Co., South Korea) equipped with flame ionization detector and Hydrodex β-6TBDM [25 m × 0.25 mm × 0.25 µm film of heptakis-(2,3-di-O-methyl-6-O-**t**-butyldimethylsilyl)-β-cyclodextrin (Macherey&Nagel)] column (oven temperature, injector and detector temperatures were 130°C, 250°C and 250°C, respectively; carrier gas: H_2 at a flow of 1.8 ml min^{-1}; split ratio: 1:50). Data on conversion, enantiomeric composition of the products [(*R*)-**2a,b** and (*S*)-**1a,b**] and the calculated properties of the enzyme are presented in Tables 8 and 9 and Figure 20.

4.3.6. General procedure for sol-gel immobilization of lipases (using binary and ternary silane precursor systems)

IPA (200 µl), TRIS-HCl buffer (0.1 M, pH 7.5, 390 µl), the imprinting additive [0.5% v/v, 5 µl: lauric acid (LA), oleic acid (OA), polyethylene glycol 400 (PEG 400), polyethylene glycol 1000 (PEG 1000), Triton X-100 (polyethylene glycol *tert*-octylphenyl ether, TRX100), BRIJ 30 (polyethylene glycol dodecyl ether, BRIJ), tetraethylene glycol (TEG), tetraethylene glycol dilauryl ester (L-TEG-L) or olive oil] and NaF solution (1M, 100 µl) were mixed in a 5 ml glass vial and the resulting solution was shaken at 1000 rpm at room temperature for 10 minutes. The corresponding silane precursors [TEOS:PhTEOS (1:1), TEOS:OcTEOS:PhTEOS (10:7:3) or TEOS:PhTEOS:DMDEOS (4:1:1); 3 mmol (in the given molar ratio)] and enzyme (50 mg) were added to the vial by continuous shaking. To complete the polymerization of the resulting in a sol suspension, the mixture was shaken for 12 hours at room temperature. The forming solid was washed with IPA (7 ml), distilled water (5 ml), IPA (5 ml) and n-hexane (5 ml). The resulting white powder was dried in a vacuum desiccator for 3 h (until 0.4 mmHg final level of vacuum). Experiments with Lipase PS and CrLwere carried out in triplicates. The sol-gel lipase preparations were stored at room temperature.

4.3.7. Scaling up the sol-gel immobilization of lipases using ternary silane precursor systems

Scale up with the general enzyme loading (1×): The general sol-gel production procedure with 20-fold enzyme amounts (1 g of Lipase AK, Lipase PS and CrL; and 1 ml of CaLB L) using ternary silane precursors [TEOS:OcTEOS:PhTEOS (10:7:3) or TEOS:PhTEOS:DMDEOS (4:1:1); 60 mmol (in the given molar ratio)] was performed with the selected imprinting additives [no additive for Lipase AK, LA (0.5 v/v%) for Lipase PS, BRIJ (0.5 v/v%) for CrL, BRIJ for

(0.5 v/v%) for CaLB]. All the other components of the general procedure were added in 20-fold amounts.

Scale up with doubled enzyme loading (2×): 20-fold amount of enzyme (1 g of Lipase AK, Lipase PS and CrL; and 1 ml of CaLB L) was added to a system containing 10-fold amounts of the components of the general procedure, the ternary silane precursors [TEOS:OcTEOS:PhTEOS (10:7:3) or TEOS:PhTEOS:DMDEOS (4:1:1); 30 mmol (in the given molar ratio)] and the selected imprinting additives [no additive for Lipase AK, LA (0.5 v/v%) for Lipase PS, BRIJ (0.5 v/v%) for CrL, BRIJ for (0.5 v/v%) for CaLB].

4.3.8. Effect of variations in the sol-formation steps for the sol-gel lipase encapsulation

Variations in the sol-formation steps of the sol-gel immobilization − mixing the silane precursors with the aqueous phase, time of the enzyme addition and the NaF initiator addition, omitting or applying ultrasonication − were investigated in triplicate with Lipase PS and CrL (for details on amounts and concentrations, see Experimental Section of the main article).

Method A: enzyme, IPA, PEG, NaF, TRIS, silane precursors were added at the same time and the mixture was shaken for 12 hours at room temperature.

Method B: IPA, PEG, NaF, TRIS and silane precursors were shaken for 5 min, then enzyme was added and the mixture was shaken for 12 hours at room temperature.

Method C: enzyme, IPA, PEG, NaF and TRIS were shaken for 5 min, then silane precursors were added and the mixture was shaken for 12 hours at room temperature.

Method D: enzyme, IPA, PEG, TRIS and silane precursors were ultrasonicated for 10 min, then NaF was added and the mixture was shaken for 12 hours at room temperature.

Method E: enzyme, IPA, PEG, NaF, TRIS, silane precursors were added at the same time ultrasonicated for 15 min, then the mixture was shaken for 12 hours at room temperature.

Based on the results of this investigation, the experiments for Lipase AK and Lipase PS were performed with Method A while in case of CaLB and CrL Method E was applied.

4.3.9. Synthesis of alkan-2-yl acetates, *rac*- 1b-g

Corresponding secondary alcohol (*rac*-**1b-g**, 0.5-0.5 g of each, individually) was mixed with acetic anhydride (2 mol equivalent) then catalytic amount of triethylamine (3-4 drops) was added then the resulting mixture was refluxed for 8-10 min. The reaction was followed by TLC (hexane:acetone 10:4).

After cooling the reaction mixture to room temperature hexane (10 mL) and water:methanol (40:60) was added. Then it was washed with saturated $NaHCO_3$ (3 × 4 mL). After drying the organic layer

on anhydrous Na_2SO_4, the solvents were removed by vacuum rotary evaporator. The standards were characterized by GC.

The NMR spectrum was recorded in $CDCl_3$ on a Bruker DRX-500 spectrometer (at 500 MHz for ^{1}H- and 125 MHz for ^{13}C-spectra).

4.3.10. Synthesis of 1-phenylethyl acetate, *rac*-2a

The solution of racemic 1-phenylethanol *rac*-**1g** (200 mg, 1.6 mmol) in hexane-*t*-BuOMe-vinyl acetate 6:3:1 (10 mL), CaLA T2-150 (200 mg) was added and the resulting mixture was shaken for 2 hours at 60°C in thermostated shaker (1100 rpm). The enzyme was filtrated, washed with EtOAc, and the solvents were removed by vacuum rotary evaporator. Light yellow liquid (257 mg, 96%).

4.3.11. Synthesis of tetraethylene glycol dilaurate (L-TEG-L)

Solution of tetraethylene glycol (3.88 g, 20 mmol) triethylamine (6.07g, 60 mmol) and anhydrous dichloromethane (200 ml) was stirred under inert atmosphere meanwhile the lauroyl chloride (10.94g, 50 mmol) was introduced to the mixture dropwise at 0°C.

The reaction was followed by TLC (hexane:acetone 10:2). The mixture was washed with 5 % HCl (2 × 60 ml), saturated $NaHCO_3$ (3 × 50 ml) and brine (2 × 50 ml). After drying on anhydrous Na_2SO_4, the solvents were removed by vacuum rotary evaporator then the residue was purified by column chromatography (Geduran® Si 60, hexane: acetone 10:0.1) to give L-TEG-L as white solid (5.86 g, 53%).

The NMR spectrum was recorded in $CDCl_3$ on a Bruker DRX-500 spectrometer (at 500 MHz for ^{1}H- and 125 MHz for ^{13}C-spectra).

4.3.12. Immobilization of Novozym CALB L from solution onto Novozym carrier

Solution of recombinant lipase B from *Candida antartica* expressed in *Aspergillus niger* (Novozym CaLB L) was immobilized on Novozym carrier in different ratios (1 mg Novozym carrier + 1 µL, 2 µL, 4 µL, 8 µL, 12 µL enzyme solutions, separately). The mixtures were shaken for 1h at 1200 rpm then kept for 8 hours at 4°C. The resulting suspensions were washed with IPA (7 ml), distilled water (5 ml), IPA (5 ml) and n-hexane (5 ml). The residual white powder was dried in a vacuum desiccator for 3 h (until 0.4 mmHg final level of vacuum). The biocatalysts obtained in this way were compared to Novozym 435. The preparations from 1:8 or 1:12 carrier:liquid enzyme ratio were found to be equivalent with Novozym 435.

4.3.13. Synthesis of 1-(thiophen-2-yl)ethanol, *rac*-1h

1-(thiophen-2-yl)ethanone (2.50 g**,** 20 mmol) was dissolved in abs. ethanol (20 mL), then $NaBH_4$ (0.38 g, 10 mmol) was added in small portions. The mixture was stirred for 30 min. The solvent was removed under vacuum. The residue was dissolved in 80 mL of EtOAc and was treated with saturated $NaHCO_3$ solution and brine. The organic layer was dried over Na_2SO_4, and the solvent was removed by vacuum rotary evaporator. Light yellowish liquid. (2.47 g, 97%).

4.3.14. Synthesis of 1-(thiophen-2-yl)ethyl acetate, *rac*-2h

1-(thiophen-2-yl)ethanol (200 mg, 1.6 mmol) was dissolved in hexane-TMBE-vinyl acetate 6:3:1, CalA T2-150 enzyme was added and shaked for 4 hours at 50°C in thermostated shaker. The enzyme was filtrated, washed with EtOAc, and the solvents were removed by vacuum rotary evaporator. Dark yellow liquid. (260 mg, 98%)

4.3.15. Characterization of the sol-gel lipase preparations using multisubstrate test systems in batch mode

The sol-gel immobilized lipase (50 mg) was added to the solution of multisubstrate mixture A (50 μL; equimolar mixture of hexan-2-ol, octan-2-ol, nonan-2-ol, decan-2-ol and dodecan-2-ol) or multisubstrate mixture B (50 μL; equimolar mixture of 1-phenylethanol and heptan-2-ol) in hexane:THF 2:1 (1 mL) or in hexane:MTBE 2:1 (1 mL) containing vinyl acetate (100 μL). The resulting mixture was shaken at 30 °C in a sealed glass vial at 1200 rpm. Samples were taken directly from the reaction mixture (sample size: 50 μL, diluted with hexane to 500 μL) at 0.5, 1, 2, 4, 8, 24 h, and analyzed by GC [Hewlett Packard 6890, Hydrodex β-6TBDM column (25 m × 0.25 mm × 0.25 μm, heptakis-(2,3-di-O-methyl-6-O-**t**-butyldimethylsilyl)-β-cyclodextrin), FID (250°C), injector (250°C), H_2 (1.8 mL min^{-1}, split ratio: 1:50). Oven (50-200 °C at 10 °C/min for mixture A; 50-90 °C at 2 °C/min, 90-130°C at 40 °C/min, and holding for 5 min for mixture B)].

ChemStation software was used to evaluate the chromatograms. Data on conversion, enantiomeric composition of the products [(*R*)-**2a-g** and (*S*)-**2a-g**] and the calculated properties of the enzyme are presented in Tables 16-18 and Figures 23-26 and in Appendix.

The enantiomer selectivity in a kinetic resolution can be calculated from two of the following three data: conversion [c], enantiomeric excess of the forming ester [ee(*R*)-2] (RAKELS et al. 1993).

4.3.16. Characterization of the sol-gel lipase preparations using multisubstrate test systems in continuous-flow reactors

The continuous-flow bioreactions were performed by laboratory flow reactor equipped with enzyme filled columns (stainless steel, inner diameter: 4 mm; total length: 70 mm; packed length: 65 mm; inner volume: 0.816 mL). Immobilized sol-gel Lipase AK, Lipase PS, CaLB and CrL were packed into stainless steel columns. For the continuous-flow enzymatic applications, the columns were sealed by silver metal filter membranes [Sterlitech Silver Membrane Filter from Sigma–Aldrich, Z623237, pore size 0.45 μm; pure metallic silver, 99.97% with no extractable or detectable contaminants]. The sealings were made of PTFE. The filling mass of the enzymes was 170-440 mg. Before use, the freshly filled columns were washed with hexane: MTBE 2:1 mixture. The solution of multisubstrate A or B/alcohols (5 mg mL^{-1}) in hexane-MTBE-vinyl acetate 6:3:1 mixture was pumped through the lipase-filled column (lipases: Lipase AK, Lipase PS, CaLB, CrL). The runs were performed at 30°C and 0.2 mL min^{-1} flow rate. Samples were collected and analyzed during stationary operation (GC at every 10 min. between 30-90 min. after changing the conditions).

4.3.17. Preparative kinetic resolution in continuous-flow mode

The solution of 1-(thiophen-2-yl)ethanol (*rac*-**1h**, 5 mg mL^{-1}, 0,039 mmol mL^{-1}) in hexane-MTBE-vinyl acetate 6:3:1 mixture was pumped through the lipase-filled column (lipases: Lipase AK, Lipase PS, CaLB, CrL, CalA T2-150). The reaction mixture was collected for 24 h, then the solvent was removed from the resulting reaction mixture in vacuum. The residue containing the mixture of the forming acetate [(*R*)-**1**] and unreacted alcohol [(*S*)-**1h**] were separated by column chromatography (Geduran® Si 60, dichloromethane). Samples were collected at 1, 2, 3, 4, 8, 24 h of operation and analyzed by GC to follow up the activity of bioreactors

5. RESULTS AND DISCUSSIONS

5.1. Specific structured lipid and phytosterol ester one-pot synthesis

Our major goal was to develop a solvent-free, purely enzymatic process for manufacturing SSLs and PSEs mixtures starting from naturally occurring starting compounds (Figure 16).

PSs (mixture of rapeseed and sunflower phytosterols, composed of brassicasterol, campesterol, stigmasterol and β-sitosterol, see it more detailed in 4.1.2.) are readily available because they are by-products of edible oil processing (CEREOL NÖVÉNYOLAJIPARI RT. 2003). Caprylic acid, a medium-chain fatty acid found in coconut, and sunflower oil as triglyceride which is rich in linoleic acid (48-74% of total FA), were used in our processes.

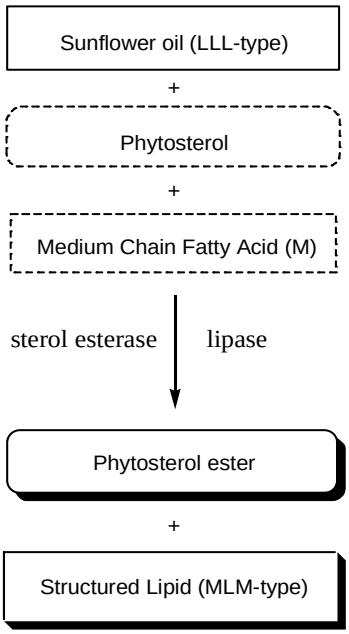


Figure 16. Enzymatic one-pot production of specific structured lipids - phytosterol esters mixture

5.1.1. Biocatalyst screening for structured lipid production and esterification of phytosterol

To find proper biocatalysts for an integrated enzymatic process leading to the desired SSLs and PSEs composition, enzyme screens were carried out first for structured lipid and sterol ester syntheses. The lipase (EC 3.1.1.13) and sterol esterase (EC 3.1.1.3) activities were tested side by side in four different reaction mixtures (Table 4). Seven commercial lipases and two sterol esterases of *Aspergillus* origin (TŐKE et al. 2007) were screened in different reactions. A mixture of sunflower oil, caprylic acid and PSs was used for testing the combined sterol esterase and lipase activity (Reaction I in Table 4). A mixture of sunflower oil and caprylic acid was used to test the lipase activities in the exchange reaction of acyl moieties of triacylglycerols with free fatty acids (Reaction II in Table 5). A third mixture of caprylic acid and PSs was used to test the sterol esterase activity in direct acylation of PSs (Reaction III in Table 6). Finally, a mixture of sunflower oil and PSs was used to test the sterol esterase activity in transesterification reaction (Reaction IV in Table

4). The Reactions I-III were tested with seven lipase and two sterol esterase preparations at three different reaction water contents (without and with addition of 0.06 and 0.12 w/w% water) whereas Reaction IV was performed only with the two *Aspergillus* SSF sterol esterase preparations and one lipase (Lipozyme) preparation.

Table 4. Reactions for biocatalyst screening

Reaction [a]	Sunflower oil (mol equiv.)	Caprylic acid (mol equiv.)	Phytosterol (mol equiv.)[c]
I[b]	2	12	1
II[b]	2	12	0
III[b]	0	12	1
IV	2	0	1

[a] The mixture was shaken for 13 days in the presence of enzymes (lipases: 100 mg, at 50 °C; SSF sterol esterases: 400 mg, at RT).
[b] Three parallel sets of experiments were performed for each reaction type without adding water and with water supplement (0.06 and 0.12 w/w%, based on the total mass of the starting compounds).
[c] Molar equivalents were based on phytosterols (414 mg, ~ 1 mmol).

Water activity is an important factor in enzymatic transesterification reactions leading to structured lipids (FOMUSO and AKOH 1997, XU 1998a), and in esterification of sterols (NAGAO et al. 2004). A certain amount of water is necessary to preserve the catalytically active conformation of the enzyme. Additional water results in a slight increase in structured lipid (SL) yield with transesterification and acidolysis reactions, since water is needed to initiate the hydrolysis before synthesis of triacylglycerols can occur. Therefore, acidolysis reactions of sunflower oil with caprylic acid (Reaction II, Table 4) and esterification reactions of phytosterol with caprylic acid (Reaction III, Table 4) were screened without added water and by addition of 0.06 % and 0.12 % water.

Table 5. Biocatalyst screening for structured lipid production

Biocatalyst	Conversion to MLM/MML (%) [a]					
	3 days [b]			7 days [b]		
	W_0 [c]	$W_{0.06}$ [c]	$W_{0.12}$ [c]	W_0 [c]	$W_{0.06}$ [c]	$W_{0.12}$ [c]
Lipase A from *Candida antarctica*	[d]	0	[d]	[d]	0.3	[d]
Novozym 435	20.7	3.6	3.6	22.8	33.8	17.7
Lipase PS	0	8.3	4.2	1.7	8.0	18.6
Lipase AK	1.7	3.0	1.4	10.8	11.7	6.7
Lipozyme	39.3	37.9	40.9	53.1	49.8	51.2
Lipozyme TL IM	2.0	4.1	1.7	10.0	7.6	4.8
Lipase from *Candida cylindracea*	0	2.4	5.1	0	7.6	18.5
SSF from *Aspergillus oryzae*	0	0.4	0	2.5	2.4	2.3

[(TŐKE et al. 2007)

[a] Conversion of sunflower oil to MLM/MML in the enzymatic reactions (type II in Table 4) was determined by GC. Besides the structured lipid (MLM/MML 0.3-53.1%) and the residual triglyceride (LLL, 0.5-99.7%), formation of LLM/LML (0.25-36.6%), and diglycerides (0-16.6%) were detected.
[b] Reaction time.
[c] W refers to the water supplement in the mixtures (0, 0.06 and 0.12 w/w%).
[d] Not tested.

In the synthesis of SLs by acidolysis with caprylic acid (Table 5), Lipozyme proved to be the most active biocatalyst resulting in 37.9 - 40.9% MLM/MML conversion at day 3, which increased further up to 53.1% after 7 days. Novozym 435, the industrial product of lipase B from *Candida antarctica,* exhibited lower activity in the acidolysis reaction of sunflower oil (17.7-33.8% conversion after 7 days). Interestingly, in the reaction with Novozym 435 without added water, the MLM/MML conversion at day 3 (20.7%) reached almost the final level (22.8% at day 7). In contrast, the MLM/MML conversions by Novozym 435 with added water remained low after 3 days (3.6%). In the case of Lipase PS and lipase from *C. cylindracea*, only moderate activity was observed in SL synthesis (18.6 and 18.5% MLM/MML conversions after 7 days, respectively). Lipase AK and Lipozyme TL IM exhibited significantly less activity (11.7 and 10.0% MLM/MML conversions after 7 days, respectively), whereas almost negligible transesterification activity was found for Lipase A from *C. antarctica* and for the SSF preparation from *A. oryzae*. The water content in the investigated range affected the acidolysis only with the biocatalysts having moderate activity (e.g. Lipase PS or lipase from *C. cylindracea*). Fortunately, the SL forming ability of the most active Lipozyme was practically independent of the amount of added water. Therefore, Lipozyme was selected as the biocatalyst for SL synthesis.

The screen for esterification of PSs with caprylic acid (Reaction III in Table 4) revealed that all of the tested biocatalysts could produce caprylic ester of PSs (Table 6). However, the final equilibrium conversion remained low with most lipases (<30% even after 7 days). In agreement with the previous results indicating that a lipase (Lip 3) of *C. rugosa* can hydrolyze sterol esters (TENKANEN et al. 2002), this enzyme proved to be the most active in PSE production (<80% after 7 days). The highest sterol ester conversion was achieved with the recently identified SSF sterol esterase of *A. oryzae* (TŐKE et al. 2007). Fortunately, water (up to 0.12 %) had no significant influence on the high final equilibrium conversion of PSE production by *Ao*SSF. Taking the operational stability into account, the highest (0.12%) water dosage was chosen for further experiments.

Table 6. Screening of biocatalysts for esterification of phytosterol with caprylic acid

Biocatalyst	Conversion to sterol ester (%) [a]					
	3 days [b]			7 days [b]		
	W_0 [c]	$W_{0.06}$ [c]	$W_{0.12}$ [c]	W_0 [c]	$W_{0.06}$ [c]	$W_{0.12}$ [c]
Lipase A from *Candida antarctica*	16.6	14.2	5.4	15.5	19.1	14.1
Novozym 435	14.1	16.3	5.9	12.9	13.9	14.5
Lipase PS	8.1	13.9	15.3	27.1	29.6	32.1
Lipase AK	9.4	19.7	11.9	14.8	17.6	12.4
Lipozyme	5.8	6.3	6.4	15.4	17.3	15.1
Lipozyme TL IM	16.5	19.0	10.0	15.8	15.4	11.2
Lipase from *Candida cylindracea*	54.5	66.0	59.5	77.7	78.2	77.0
SSF from *Aspergillus oryzae* [(TŐKE et al., 2007)	76.1	91.3	91.2	96.2	95.7	96.7

[a] Conversions of phytosterols to their caprylic esters by enzymatic reactions (type III in Table 4) were determined by GC.
[b] Reaction time
[c] W refers to the water supplement in the mixtures (0, 0.06 and 0.12 w/w%).

According to the experiments with caprylic (C_8) and lauric (C_{12}) acid, there was only a slight difference in the fatty acid chain length preference of the SSF sterol esterase from *A. oryzae* in the PSE formation (91.2% conversion with caprylic acid and 94.9% with lauric acid after 3 days). Independently from the chain length, similar equilibrium conversions were reached (96.7% with caprylic acid and 95.1% with lauric acid after 7 days).

Sterol esterase activity was also tested in the transesterification reactions of sunflower oil with PSs (reaction type IV in Table 4) resulting in formation of long-chain PSEs. After two days of incubation, both SSF *Aspergillus* sterol esterases (TŐKE et al. 2007) exhibited good conversions (83.6% with *Ao*SSF and 85.7% with *As*SSF) which were further increased after eight days (89.4% with *Ao*SSF and 88.9% with *As*SSF). Based on these screen results, the SSF sterol esterase preparation of *A. oryzae* was selected for the production of PSEs.

After the preliminary tests, several variations of the one-pot processes were performed (Table 7). Most of the PSE conversion occurred in 3 to 4 days, but the final conversion required longer reactions (Table 6). In addition, the reverse hydrolysis of the PSEs was a rather slow process either by the *Ao*SSF biocatalyst or by Lipozyme. The SL formation with Lipozyme required more than 3 days as well (Table 5), but this process was more reversible. Therefore, the PSE formation by the *Ao*SSF biocatalyst was chosen as the first enzymatic reaction in all process variations (A-F Table 7).

Table 7. Enzymatic one-pot production of specific structured lipid - phytosterol ester mixtures

Process [a]	1st reaction: biocatalyst [b]; time (day)	Water removal / incubation [c] (day)	Filtration of *Ao*SSF	2nd reaction: biocatalyst [b]; time (day)	Total reaction time (day)
A	*Ao*SSF; 3	+ / 1	-	*Ao*SSF/Lip; 4	8
B	*Ao*SSF; 3	+ / 1	+	Lip; 4	8
C	*Ao*SSF; 4	-	-	*Ao*SSF/Lip; 4	8
D	*Ao*SSF; 3	+ / 1	-	-	4
E	*Ao*SSF/Lip; 4	-	-	-	4
F	*Ao*SSF/Lip; 3	+ / 1	-	-	4

[a] All processes were carried out in 3 parallel series with different caprylic acid amounts [molar ratios of phytosterol:sunflower oil:caprylic acid; series 1: 1:2:12 (type I in Table 4); series 2: 1:2:18 and series 3: 1:2:24].
[b] All processes were carried out at 50°C and 1200 rpm using SSF sterol esterase from *Aspergillus oryzae* (*Ao*SSF, 400 mg) with or without addition of Lipozyme (Lip, 100 mg).
[c] The forming water was removed by rotary evaporation (7 min at 50°C and 30 mmHg) and the mixture was incubated for 1 more day at 50°C and 1200 rpm.

All process variations were tried in three parallel series using increasing amounts of caprylic acid [the ratio of PS:sunflower oil:caprylic acid was 1:2:12, 1:2:18 and 1:2:24 in Series 1, 2 and 3, respectively (processes A-F in Figure 17)]. Increasing amounts of caprylic acid (Series 2 and 3 processes) resulted in similar or even slightly lower conversions to PSE or SLs than achieved at the lowest caprylic acid concentration (Series 1 processes).

Several two-step processes by sequential addition of the *Ao*SSF biocatalyst and Lipozyme were tried first (Processes A-C in Table 7). In Process A, the reaction mixture was incubated with the *A. oryzae* SSF biocatalyst for 3 days. Although no significant influence of the amount of added water on PSE formation by *Ao*SSF (Table 6) or structured lipid formation by Lipozyme (Table 5) were found, water was removed by vacuum treatment after 3 days followed by incubation with the *Ao*SSF biocatalyst for an additional day. After addition of Lipozyme, the reaction mixture was incubated for 4 more days. Analysis of the time course of the SSLs - PSEs mixture formation revealed (A1 process in Figures 17 and 18) that PSE formation reached almost 80% after the first 3 days. According to our expectations from the preliminary experiments, PSEs were derived mostly from direct acylation with caprylic acid rather than transesterification with the sunflower oil. The water removal and the further 5 days of incubation increased the PSE conversion up to 92.1%. Not surprisingly, significant SL formation occurred only after addition of Lipozyme at day 4. The final MLM/MML conversion reached 44.1% by this process. The most prominent components, besides the forming PSEs and SLs, were MMM/MLL/LML type triacylglycerols, residual PSs (7.9 %) and various diglycerides (~6 %). According to several studies (KAO CORPORATION 1999), however, fat formulations containing diglycerides and PSs also have beneficial health effects.

Two further variants of the sequential addition of the *A. oryzae* SSF biocatalyst and Lipozyme were tried next (Processes B and C in Table 7). In Process B, the *Ao*SSF biocatalyst was removed

by filtration after the first 3 days incubation period and water removal, and the filtrate was incubated with Lipozyme for 4 days. As expected, this process (B1 process in Figure 17) resulted in lower final PSE content than Process A. In Process C, water removal at day 3 was omitted. Again, slightly less PSEs and SLs were formed than in Process A (C1 Process in Figure 17).

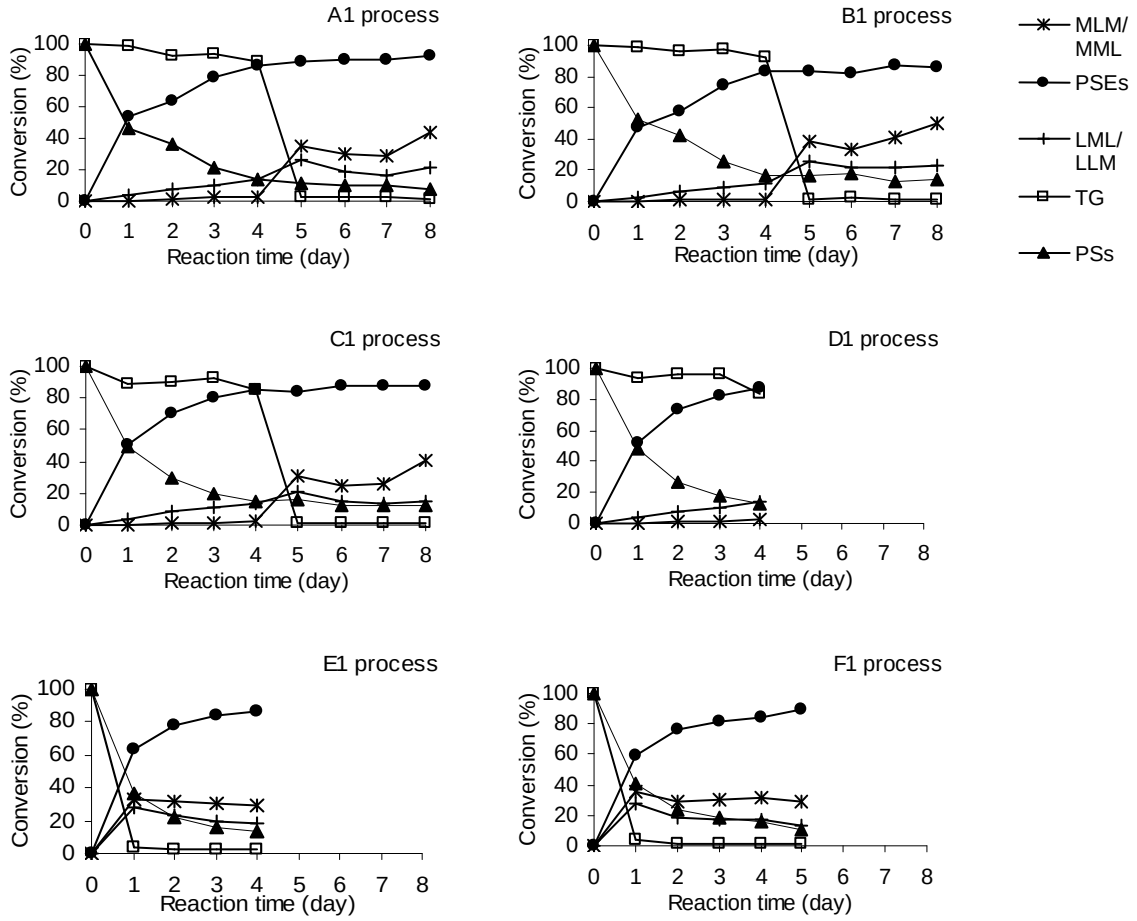


Figure 17. Time course of specific structured lipids - phytosterol esters mixture formation by enzymatic one-pot processes (series 1 for the A-F processes are shown)

Next, shortening the processes was tried (Processes D-F in Table 7 and D1-F1 in Figure 17). Omitting the Lipozyme (Process D in Table 4) resulted in a mixture containing low amounts of SLs (MLM/MML) (D1 in Figure 17). Co-addition of the *Ao*SSF biocatalyst and Lipozyme without (Process E in Table 4) or with water removal after 3 days (Process F in Table 7) resulted in reduced amounts of PSEs (86.3% and 84.7%, respectively, instead of 92.1% in Process A1,) and SLs (29.0% and 31.4%, respectively, instead of 44.1% in Process A1) compared to process A but required only 4 days.

The best final compositions derived from the two-step processes started with addition of the *Ao*SSF biocatalyst followed by with intermediate water removal and further reaction with addition of Lipozyme (processes A1 and B1). The final mixtures from these reactions contained mostly the

desired PSEs (21.5-22.5 w/w%) and MMM/LLM/LML/LLL triacylglycerols (14-17 w/w%), besides the beneficial MLM/MML triacylglycerols (13.5-17 w/w%), diglycerides (2.5-3.5 w/w%), residual PSs (1.5-2.5 w/w%) and excess caprylic acid (36-37 w/w%).

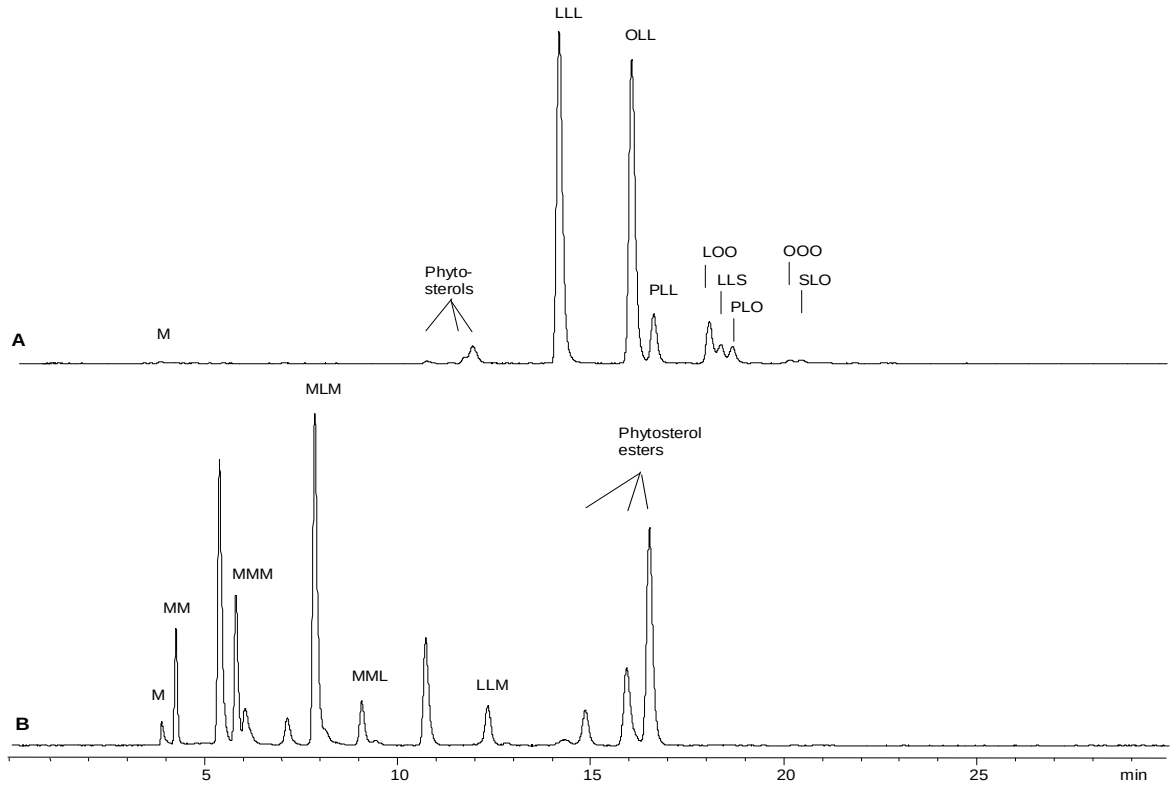


Figure 18. HPLC chromatograms of the (A) starting compounds and (B) the product mixture of the A1 process

According to our processing experiments, after simple filtration of the biocatalysts from the reaction products, the free caprylic acid can be easily removed by a simple vacuum distillation carried out under nitrogen atmosphere. Modelling experiments indicated that scale-up of the processing method from 4 g to 100 g scale is feasible. The MMM/LLM/LML triacylglycerols, which cannot be removed by this process, may be considered as neutral or slightly beneficial from nutritional point of view (HELLNER et al. 2010).

Chemically transesterified compositions of SLs and PSEs with health and nutrition promoting characteristics similar to our enzymatically produced final product (after free fatty acid removal) were patented by a multinational company (BUNGE OILS INC. 2005, 2007), because these compositions could be attractive for consumers and potentially suitable for food applications.

5.2. Improving robust sol-gel immobilized biocatalysts

As it was proved earlier in our research group that sol-gel process is an easy and effective way to create robust biocatalysts with improved stability and activity thus it was selected for further optimization (PÉTER et al. 2005).

Because of the possibility of comparison of our systematic series of experiments–in which precursors, carriers, immobilization conditions were varied and impacts of additives was evaluated - the experiments were carried out on lipase derived from *Pseudomonas fluorescens* (Lipase AK). The method (normal and composite sol-gel respectively) was extended for Lipase PS lipases and different carriers as well.

Our goal was to perform a systematic study of the sol-gel immobilization of a Celite-supported lipase using ternary silane precursor systems of various alkyltriethoxysilanes (R'-$Si(OEt)_3$ /alkylTEOS), phenyltriethoxysilane ($PhSi(OEt)_3$ /PhTEOS) and tetraethoxysilane ($Si(OEt)_4$ /TEOS).

Our earlier results showed that biocatalysts gained by sol-gel immobilization after pre-adsorption of the enzyme onto a solid support (Celite® 545), had numerous beneficial properties (e.g. the particle size distribution of the carrier remained almost the same, specific activity of the immobilized enzyme has increased by one order, furthermore when a CatCart™ column was filled with the Celite supported sol-gel Lipase AK, it could be still effectively used to catalyze acylation reactions) (TOMIN et al. 2010b). By virtue of these experiences the new biocatalysts were developed applying composite sol-gel systems.

The prepared biocatalysts were tested in kinetic resolution reaction of the simplest aromatic racemic secunder alcohol, 1-phenylethanol (*rac*-**1a**). For comparison, heptan-2-ol, *rac*-**1b** as a moderate racemic secunder alcohol was tested in acetylation reactions (CSAJÁGI et al. 2008) (Figure 19).

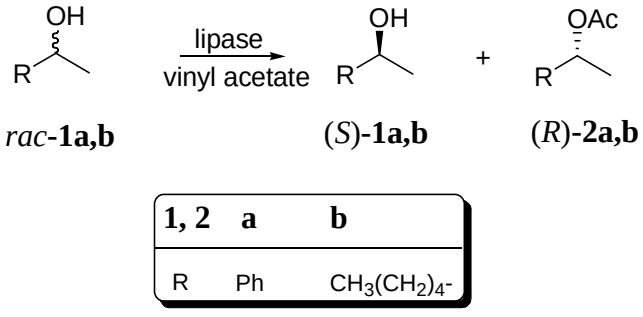


Figure 19. Kinetic resolution of racemic secondary alcohols *rac*-**1a,b** as test reaction for characterization of the free and immobilized Lipase AK biocatalysts

In order to evaluate the efficiency of the immobilization and biocatalysts, conversions (c) specific activities (U_B), activity yields (Y_A) and enantiomer selectivities (E) were compared. As the activities in the present work were calculated from a single time-point data, the term "activity" must

be considered only for comparative evaluation of the catalytic efficiencies, not for the real kinetic behavior of the biocatalyst. The immobilization efficiency was evaluated by calculating the percentage of supplied protein encapsulated in the gel.

The specific activity of the biocatalyst [U_B ($\mu mol \times min^{-1} \times g^{-1}$)] could be determined in the test reaction from the amount of the racemic alcohol [n_{rac} (μmol)], the conversion [c], the reaction time [t (min)] and the mass of the sol-gel immobilized biocatalysts [m_B (g)].

$$U_B = (n_{rac} \times c) / (t \times m_B)$$

As the activities in the present work were calculated from single time-point data, the term "activity" must be considered only for comparative evaluation of the catalytic efficiencies, not for the real kinetic behavior of the biocatalyst.

The activity yield [Y_A (%)] can be calculated from specific activity of the free Lipase AK (U_{LAK}) compared to the specific activity of the enzyme content of immobilized biocatalyst ($U_{imm\text{-}LAK}$). $U_{imm\text{-}LAK}$ can be determined in the test reaction from the amount of the racemic alcohol [n_{rac} (μmol)], the conversion [c], the reaction time [t (min)] and the mass of the lipase immobilized in the sol-gel biocatalysts [m_E (g)].

$$Y_A = 100 \times U_{LAK} / U_{imm\text{-}LAK} = 100 \times U_{LAK} / [(n_{rac} \times c) / (t \times m_E)]$$

The enantiomer selectivity in a kinetic resolution can be calculated from two of the following three data: conversion [c], enantiomeric excess of the remaining alcohol [$ee_{(S)\text{-}1}$], enantiomeric excess of the forming ester [$ee_{(R)\text{-}2}$] (CHEN et al. 1982, RAKELS et al. 1993).

5.2.1. Fine tuning of sol-gel immobilization, selection of the lipase, support and silane precursors

Binary sol-gel systems of trialkoxy- and tetraalkoxysilane precursors [R'-$Si(OR)_3$ and $Si(OR)_4$] (REETZ et al. 1995, KAWAKAMI and YOSHIDA 1996) but ternary sol-gel systems of a tetraalkoxysilane and two different trialkoxysilane precursors [R^1-$Si(OR)_3$, R^2-$Si(OR)_3$ and $Si(OR)_4$] had not been studied for lipase immobilization.

The lipase from *Pseudomonas fluorescens* (Lipase AK) proved to be a good lipase model for studies of factors influencing the sol-gel immobilization of lipases (PÉTER et al. 2005, ZARCULA et al. 2009). The properties of Celite-supported Lipase AK immobilized with ternary silane precursor systems of various alkyltriethoxysilanes (alkylTEOS), phenyltriethoxysilane (PhTEOS) and tetraethoxysilane (TEOS) were investigated in this work.

5.2.2. Adsorption of Lipase AK on Celite

A two-step immobilization protocol involving pre-adsorption of the enzyme onto solid support followed by sol-gel entrapping was applied. Thus, to select the ideal enzyme distribution on

the solid support in the first step, various amounts of Lipase AK were adsorbed on Celite 545 as solid support by precipitation from aqueous solution with slow addition of cold acetone.

Celite, also known as Diatomite or Kieselgur, is a naturally occurring, inexpensive fossilic diatomaceous earth. It consists of fossilized remains of diatoms, a type of hard-shelled algae, ranging in size from ca. 2-200 μm (HASLE and SYVERSTEN 1997), that are composed of a cell wall comprising silica (HORNER 2002). This siliceous wall can be highly patterned with a variety of pores, ribs, minute spines, marginal ridges and elevations.

To study the surface coverage of Celite, supported lipase AK preparations of different enzyme-Celite ratios (1:10, 1:4, 1:2, 3:4 and 1:1) were made. The free Celite (Figure 20A), free Lipase AK (Figure 20B), a sol-gel entrapped Lipase AK on Celite (Figure 20C) and Celite preparations covered with various amounts of Lipase AK (Figure 20D-H) were inspected by scanning electron microscopy (SEM).

The SEM investigations indicated that the commercial Lipase AK consists of relatively large spherical aggregates (4-40 μm diameter, Figure 20B). This size distribution indicates that substantial diffusion limitation may occur when the free form of Lipase AK is used for catalysis in organic media. Visual inspection of the SEM images of the free Celite revealed that the disc-shaped particles in Celite (Figure 20A) are the best forms to investigate the coverage of the solid support (Figure 20C-H).

The SEM pictures indicated that at increased amounts of the precipitated enzyme on Celite more and more monoclinic or orthorhombic particles were formed on the surface of the support (Figure 20D-H). It is known that lipases from different *Pseudomonas* strains crystallize in similar crystal forms; for example the lipase from *P. cepacia* (PDB code: 1YS1) forms monoclinic crystals (MEZZETTI et al. 2005), while the lipase from *P. aeruginosa* (PDB code: 1EX9) has orthorombic crystal structure (NARDINI et al. 2000). Because the lipase from *P. fluorescens* (Uniprot code A9YY76) exhibits high amino acid sequence homology with lipase of *P. cepacia* (37% identity, 53% homology) and *P. aeruginosa* (45% identity, 60% homology), it can be supposed that Lipase AK of *P. fluorescens* origin forms similar crystals (typically of 1-5 μm size, Figure 20F-H) as lipases from *P. aeruginosa* or *P. cepacia*. It is then understandable that the specific activity of the enzyme decreases at higher enzyme loading due to the increasing diffusion limitation within these relatively large crystals. According to the morphology studies, the 1:10 enzyme-Celite ratio provided thin and uniform coating on the surface of Celite (Figure 20D) and was selected for the further investigations. The SEM image of one of the best performing ternary sol-gel immobilized preparations (Figure 20C; Celite:Lipase AK 10:1, entrapped in octylTEOS/PhTEOS/TEOS 0.7/0.3/1 sol-gel) indicated that surface coating of Celite remained uniform and thin after the sol-gel entrapping step as well.

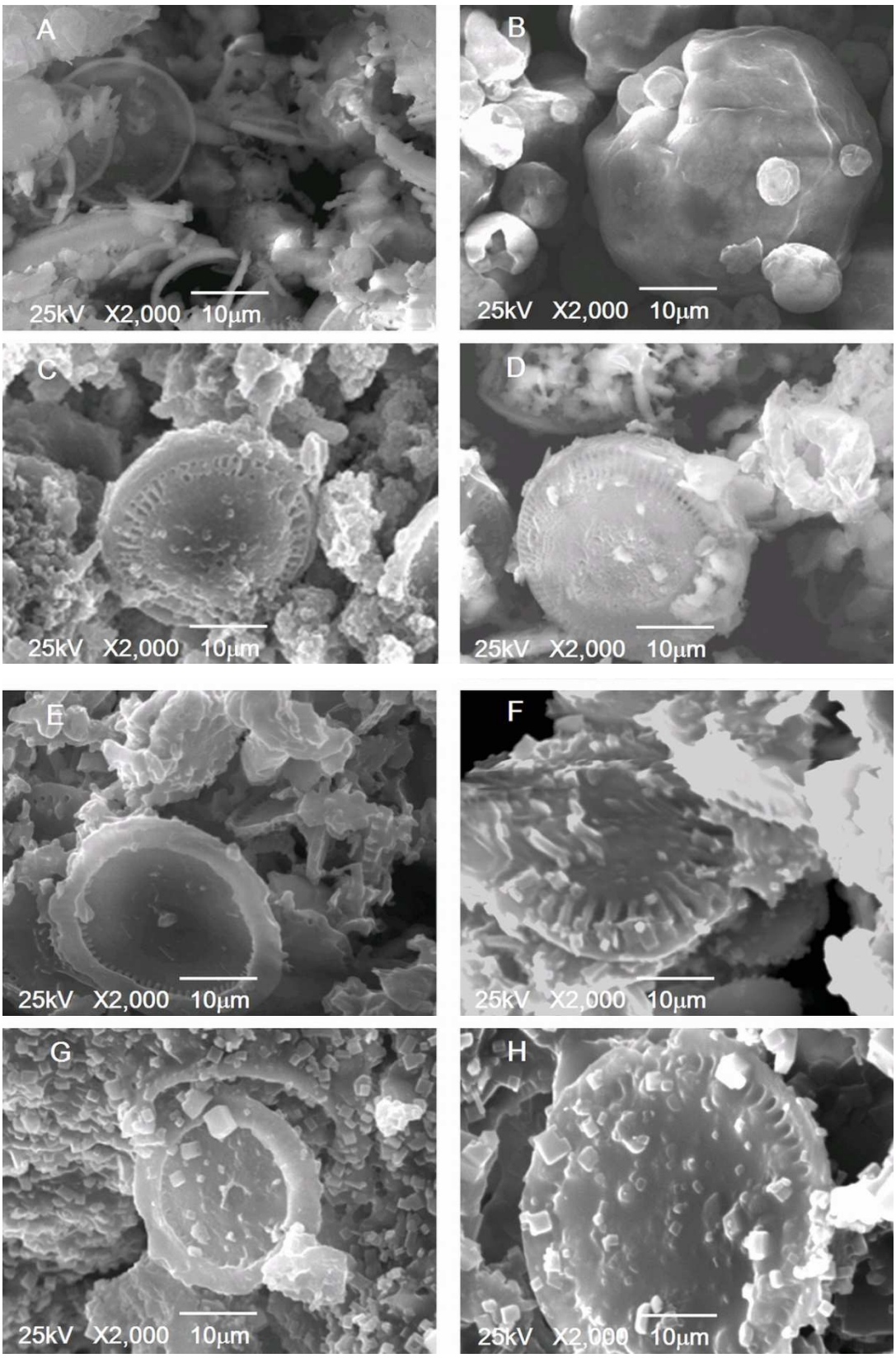


Figure 20. SEM images of Celite (A), free Lipase AK (B), Celite supported Lipase AK immobilized in octylTEOS/PhTEOS/TEOS 0.7/0.3/1 sol-gel (C) and various amounts of Lipase AK precipitated onto Celite [Celite/Lipase AK ratio: 10:1 (D), 4:1 (E), 10:5 (F), 4:3 (G) and 1:1 (H)].

5.2.3. Binary precursor matrices

The effect of the silane precursor composition on enantiomer selectivity and catalytic ability were investigated in the kinetic resolution of racemic secondary alcohols (Figure 18). All binary and ternary sol-gel preparations were tested with the selective acylation of 1-phenylethanol (*rac*-**1a**), in hexane-THF using vinyl acetate as acyl donor (Figure 20, Panels A in Tables 8 and 9). Acylation of racemic 2-heptanol (*rac*-**1b**), exhibiting moderate enantiomer selectivity with free Lipase AK, was also investigated with the binary (Panel B of Table 8) and selected ternary sol-gel preparations (Panel B of Table 9). To evaluate the efficiency of the immobilization and biocatalysts, the specific activities (U_B), and activity yields (Y_A) and enantiomer selectivities (*E*) with substrate *rac*-**1a** were compared at ~30% conversion in all cases. The enantiomer selectivity (*E*) and activity of the free Lipase AK under identical reaction conditions and conversion ranges were the references in all cases.

It had already been indicated that mixtures of R'-Si$(OEt)_3$ and Si$(OEt)_4$ at 1:1 molar ratio provided ideal matrices for the sol-gel process (REETZ et al. 2003). Accordingly, the binary systems [R-Si$(OEt)_3$:TEOS=1:1] of eight different triethoxysilanes (PrTEOS, HexTEOS, OctTEOS, PFOctTEOS, DecTEOS, DodTEOS, OctdTEOS, PhTEOS) were investigated first in the acylation reaction of 1-phenylethanol (*rac*-**1a**, Panel A of Table 8) and 2-heptanol (*rac*-**1b**, Panel B of Table 8). In the cases of HexTEOS, PFOctTEOS and PhTEOS, the activity yield (Y_A) for the binary sol-gel entrapped Lipase AK exceeded 100% with both test alcohols (Table 8). The best enantiomer selectivities (*E*) were achieved with the binary systems of PrTEOS, OctTEOS, DodTEOS and PhTEOS for *rac*-**1a** (Panel A of Table 8) and with the binary sol-gel biocatalysts containing HexTEOS, OcTEOS, PFOctTEOS and PhTEOS for *rac*-**1b** (Panel B of Table 8).

From the data with the R-Si$(OEt)_3$:TEOS=1:1 systems, it was obvious that among the binary systems the PhTEOS:TEOS=1:1 composition resulted in optimal properties regarding both activity and selectivity.

Table 8. Characterization of Celite supported Lipase AK immobilized in binary sol-gel systems of alkylTEOS/TEOS silane precursors. Specific activity (U_B), activity yield (Y_A) and enantiomer selectivity (E) of the free and immobilized forms of Lipase AK are shown in Panel A (with 1-phenylethanol *rac*-**1a**) and Panel B (with 2-heptanol *rac*-**1b**).

No.	Silane precursors[a]	Substrate	Time (h)	c (%)	E[b]	U_B (U g^{-1})	Y_A (%)
Panel A							
1	- (Lipase AK)	*rac*-**1a**	0.25	28	»200	154.2	100
2	PrTEOS:TEOS	*rac*-**1a**	8	24	»200	4.1	60
3	HexTEOS:TEOS	*rac*-**1a**	2	28	>200	19.0	181
4	OctTEOS:TEOS	*rac*-**1a**	4	25	»200	8.4	77
5	PFOctTEOS:TEOS	*rac*-**1a**	2	24	>200	16.8	156
6	DecTEOS:TEOS	*rac*-**1a**	8	33	>200	5.7	54
7	DodTEOS:TEOS	*rac*-**1a**	8	27	»200	4.6	44
8	OctdTEOS:TEOS	*rac*-**1a**	8	29	>200	5.0	44
9	PhTEOS:TEOS	*rac*-**1a**	2	39	»200	26.8	242
Panel B							
10	- (Lipase AK)	*rac*-**1b**	1	28	13.8	38.4	100
11	PrTEOS:TEOS	*rac*-**1b**	8	0.3	1.2	0.1	7
12	HexTEOS:TEOS	*rac*-**1b**	8	17	12.6	2.9	109
13	OctTEOS:TEOS	*rac*-**1b**	8	8	10.8	1.3	49
14	PFOctTEOS:TEOS	*rac*-**1b**	8	22	12.2	3.7	139
15	DecTEOS:TEOS	*rac*-**1b**	8	8	11.0	1.3	51
16	DodTEOS:TEOS	*rac*-**1b**	8	4	10.6	0.6	25
17	OctdTEOS:TEOS	*rac*-**1b**	8	5	10.0	0.9	32
18	PhTEOS:TEOS	*rac*-**1b**	8	30	14.7	5.2	187

[a] Lipase AK was preadsorbed on Celite 545 at 1:10 enzyme:support ratio and entrapped with alkylTEOS:TEOS silane precursors at 1:1 molar ratio.

[b] The enantiomer selectivity (E) was calculated from c and $ee_{(S)\text{-}\mathbf{1}}$ / $ee_{(R)\text{-}\mathbf{2}}$ (CHEN et al. 1982 and RAKELS et al. 1993). Due to sensitivity to experimental errors, E values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

5.2.4. Ternary precursor systems

The goal of this work was to provide an effective sol-gel immobilization method from an economical point of view, therefore the fine-tuning was achieved using ternary precursor system (TEOS:OcTEOS:PhTEOS:) (Depicted on Figure 21 with black).

Next, by keeping the beneficial PhTEOS as one of the three silane precursors, we tried fine-tuning of the sol-gel system by using ternary systems of alkylTEOS:PhTEOS:TEOS silane precursors in various ratios. Thus, the alkylTEOS:PhTEOS molar ratio was varied from 0.1 to 0.9 in 0.1 steps while keeping the trialkoxysilane (alkylTEOS:PhTEOS) : tetraalkoxisilane (TEOS) molar ratio at 1:1. The properties of the resulting series of ternary sol-gel biocatalysts were characterized by testing their catalytic behavior in the kinetic resolution of 1-phenylethanol *rac*-**1a** (Figure 3). The specific activity (U_B), activity yield (Y_A) and enantiomer selectivity (E) values of the enantiomer selective acetylation of racemic 1-phenylethanol *rac*-**1a**, calculated at the initial part of the reaction (at 4 h), were compared for the ternary sol-gel Lipase AK biocatalysts, the PhTEOS:TEOS binary sol-gel immobilized Lipase AK and free Lipase AK. Comparison of the ternary systems to the best binary biocatalyst (PhTEOS:TEOS=1:1) at 4 h indicated that properties

of numerous ternary compositions were superior to the PhTEOS:TEOS binary system in all aspects (Figure 21). In general, the U_B and Y_A values, related to the productivity of the biocatalysts with racemic 1-phenylethanol *rac*-**1a**, were the best for ternary compositions prepared from medium chain alkylsilane precursors (HexTEOS, OctTEOS, PFOctTEOS), while enantiomer selectivities (*E*) were sufficient for almost all longer alkylTEOS precursors (HexTEOS, OctTEOS, PFOctTEOS, DecTEOS, DodTEOS, OctdTEOS). Among all ternary systems, the perfluorinated chain containing PFOctTEOS series exhibited the best overall performance. Taking the price of PFOctTEOS also into account, however, the OctTEOS series provided the best performance / price result in the kinetic resolution of 1-phenylethanol *rac*-**1a**.

Table 9. Characterization of the best performing Celite supported ternary sol-gel Lipase AK biocatalysts prepared from alkylTEOS/PhTEOS/TEOS silane precursors. Specific activity (U_B), activity yield (Y_A) and enantiomer selectivity (E) of the free and immobilized forms of Lipase AK are shown in Panel A (with 1-phenylethanol *rac*-**1a**) and Panel B (with 2-heptanol *rac*-**1b**)

No.	Triethoxy silane precursors[a]	Substrate	AlkylTEOS amount[b] (%)	Time (h)	c (%)	E[c]	U_B (U g^{-1})	Y_A (%)
Panel A								
1	- (Lipase AK)	*rac*-**1a**	-	0.25	28	»200	154.2	100
2	PrTEOS:PhTEOS	*rac*-**1a**	60	8	25	»200	4.3	52
3	HexTEOS:PhTEOS	*rac*-**1a**	60	4	35	>200	12.2	116
4	OctTEOS:PhTEOS	*rac*-**1a**	70	4	34	»200	11.8	109
5	PFOctTEOS:PhTEOS	*rac*-**1a**	90	2	24	»200	16.7	181
6	DecTEOS:PhTEOS	*rac*-**1a**	50	8	27	»200	4.6	18
7	DodTEOS:PhTEOS	*rac*-**1a**	30	2	20	»200	13.6	53
8	OctdTEOS:PhTEOS	*rac*-**1a**	60	4	30	»200	10.2	99
9	PhTEOS	*rac*-**1a**	0	2	39	»200	26.8	242
Panel B								
10	- (Lipase AK)	*rac*-**1b**	-	1	28	13.8	38.4	100
11	PrTEOS:TEOS	*rac*-**1b**	60	8	4.7	11.6	0.8	40
12	HexTEOS:TEOS	*rac*-**1b**	60	8	6	10.2	1.0	35
13	OctTEOS:TEOS	*rac*-**1b**	70	8	19	12.3	3.2	64
14	PFOctTEOS:TEOS	*rac*-**1b**	90	8	27	12.3	4.7	205
15	DecTEOS:TEOS	*rac*-**1b**	50	8	2.6	8.3	0.1	32
16	DodTEOS:TEOS	*rac*-**1b**	30	8	21	13.5	3.6	128
17	OctdTEOS:TEOS	*rac*-**1b**	60	8	16	15.6	2.8	108
18	PhTEOS	*rac*-**1b**	0	8	30	14.7	5.2	187

[a] Lipase AK was preadsorbed on Celite 545 (1:10 enzyme:support ratio) and entrapped in a sol-gel from alkylTEOS:PhTEOS:TEOS silane precursors at x:(100-x):100 molar ratio.

[b] Amount (x) of alkylTEOS in the triethoxysilane mixture.

[c] The enantiomer selectivity (E) was calculated from c and $ee_{(S)\text{-}\mathbf{1}}$ / $ee_{(R)\text{-}\mathbf{2}}$ (CHEN et al. 1982 and RAKELS et al. 1993). Due to sensitivity to experimental errors, E values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

Next, the OctTEOS series and a well performing member of each other alkylTEOS ternary series (marked in black in Figure 20) were investigated in the kinetic resolution of 2-heptanol *rac*-**1b**, a moderate substrate of Lipase AK. Comparison of the data from the kinetic resolutions of the two different secondary alcohols, 1-phenylethanol *rac*-**1a** and 2-heptanol *rac*-**1b** with the binary (Table 8) and the selected ternary (Table 9) sol-gel systems revealed that different silane precursor compositions resulted in the best activities and selectivities for the two substrates *rac*-**1a,b** (Panels A for *rac*-**1a** and Panels B for *rac*-**1b** in Tables 8 and 9, respectively).

In the case of the kinetic resolution of 1-phenylethanol *rac*-**1a** at around 30% conversion (Panels A in Tables 8 and 9), the addition of PhTEOS to longer chain alkylTEOS (OctTEOS, PFOctTEOS, DecTEOS, DodTEOS, OctdTEOS) resulted in ternary sol-gel systems with enhanced activity yield (Y_A) and enantiomer selectivity (*E*) (Entries 4-8 in Table 9) compared to the corresponding binary systems (Entries 4-8 in Table 8).

In the case of the kinetic resolution of 2-heptanol *rac*-**1b**, encapsulation in all alkylTEOS containing binary systems resulted in decreased selectivity (Entries 11-17 in Table 1, *E*= 1.2 – 12.6) compared to the free Lipase AK (Entry 10 in Table 8, *E*= 13.8). Only the binary PhTEOS system had a slight selectivity enhancement in acylation of *rac*-**1b** (Entry 18 in Table 8, *E*= 14.7). Not surprising, that addition of PhTEOS to alkylTEOS in the ternary systems enhanced the selectivity of the encapsulated lipase in all cases except DecTEOS (Panel B in Table 9). In the ternary systems, the specific activity (U_B) and activity yield (Y_A) were also significantly improved (Panel B in Table 9) compared to the pure alkylTEOS containing binary systems (Panel B in Table 8) with only two exceptions, HexTEOS and DecTEOS (Entries 12 and 15 in Table 9). As found earlier with 1-phenylethanol *rac*-**1a**, there were ternary systems that surpassed the selectivity (*E*= 15.6, Entry 17 in Table 9) or productivity (Y_A= 205 %, Entry 14 in Table 9) of the pure binary PhTEOS:TEOS system (Entry 18 in Table 8) in the kinetic resolution of 2-heptanol *rac*-**1b** as well.

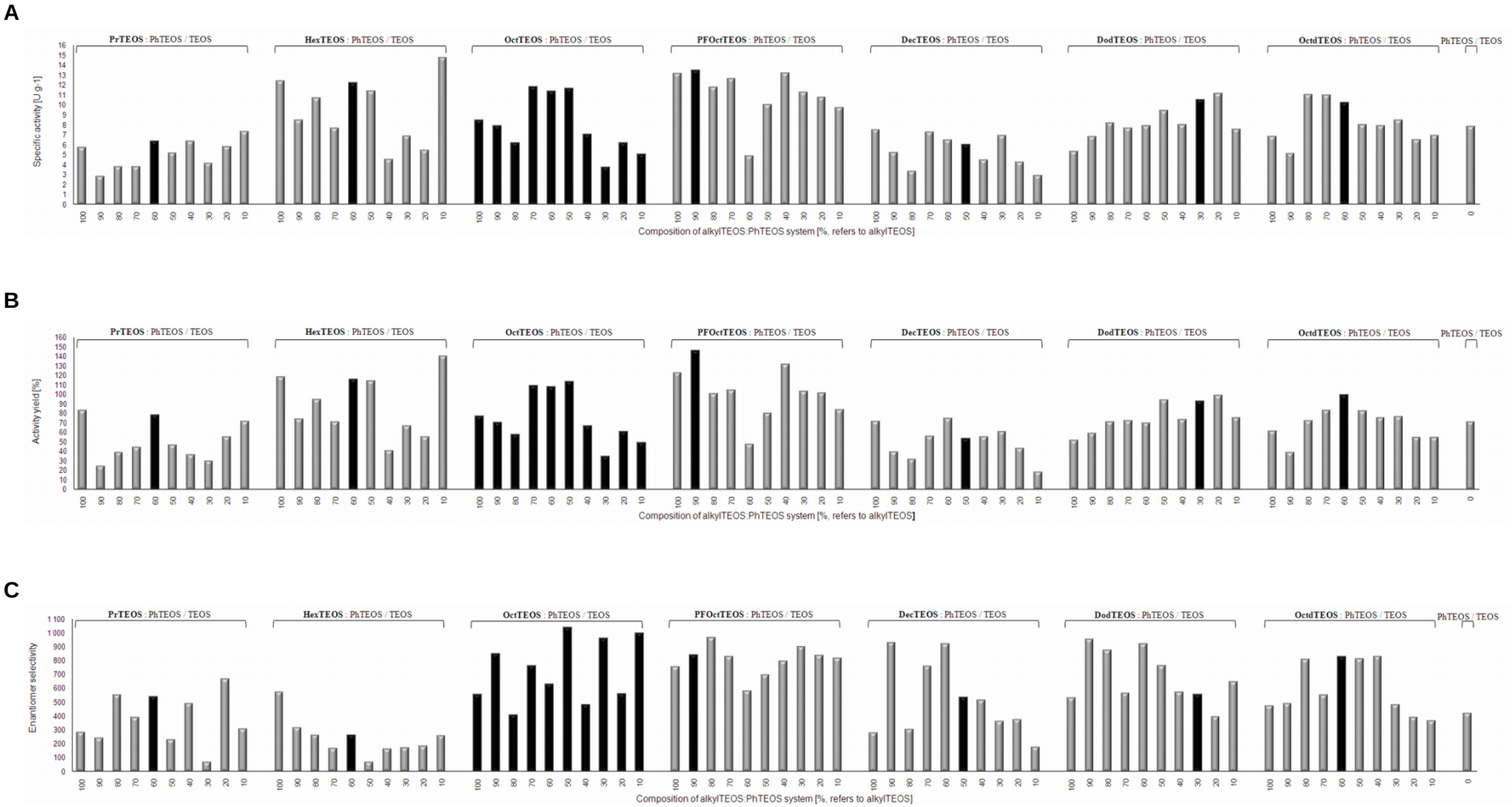


Figure 21 Characterization of Celite supported ternary sol-gel Lipase AK biocatalysts prepared from alkylTEOS/PhTEOS/TEOS silane precursors. Specific activity, U_B (A); activity yield, Y_A (B) and enantiomer selectivity, E (C) of the free and immobilized forms of Lipase AK are calculated at 4 h reaction time for enantiomer selective acetylation of racemic 1-phenylethanol *rac*-**1a**. The octylTEOS series and representatives of the other alkylTEOS systems included in Table 9 are shown in black.

5.2.5. Extension of sol-gel immobilization system

Additionally important parameters influencing the activity of sol-gel encapsulated lipases were investigated. Among others two different carriers namely Celite® 545 and Geduran silica gel was compared (Table 10), furthermore the enzyme: carrier ratio were varied (1: 5 and 1:10) (Table 10) with three different lipases, Lipase AK (Table 10 and 12), Lipase PS (Table 11 and 13) and CrL (Table 14).

The composite-type immobilization were performed in two ways; in one case the enzyme was precipitated by acetone on the surface of the support material followed by drying step before sol-gel immobilization while in the other procedure during gelation the enzyme and the support material were added at the same time to the sol system and it was intensively mixed and shaken. Based on our preliminary results the best binary (TEOS: PhEOS; 1:1) and ternary (TEOS: OcTEOS:PhTEOS; 10:7:3) sol-gel systems were used in this study.

The efficiency of the prepared biocatalysts can be characterized by the same parameters (were described earlier) as specific activity (U_B), specific enzyme activity (U_E) and enantiomer selectivity (E).

5.2.5.1. The influence of particle size of carrier molecule and enzyme/carrier ratio on biocatalysts' activity

The success and efficiency of the physical adsorption of the enzyme on a solid support depends on several parameters. The size of protein to be adsorbed, the specific area of the carrier and the nature of its surface (porosity and pore size) are crucial. The use of porous support is advantegous since the enzyme can be adsorped at the outer surface of the material and within the pores as well. Immobilization of an enzyme in a porous carrier usually follows the moving front theory; the enzyme molecules first occupy the outer shell of the carrier and move slowly to the interior of the carrier (CAO 2005b).

Celite has lower porosity thus the immobilization probably occurs on its surface resulting in a monolayer type immobilization. While in case higher porosity silica gel the enzyme molecules can reach the interior pores as well.

In general, three types of enzyme distribution on carriers can be distinguished: convex-type (inclomplete adsorpion), uniform-type (complete adsorption) and concave-type ("overloaded"). The activity expression is the highest with convex distribution thus it is suitable for enzymatic reactions suffer from diffusion constraints (CAO 2005b).

Based on our experiments it can be observed that the immobilization on carrier has a beneficial effect on the behavior of biocatalyst. Immobilization with preadsorption (enzyme precipitated by acetone on the carrier) resulted in increased activity (Tables 10-14).

With applying higher porosity silica gel it was managed to exceed the results of Celite® 545even without preadsorption. It seems in case of Lipase AK with the lower amount of enzyme (1/5) demonstrated the concave-type immobilization and thus mitigated the diffusion constraints as it was resulted in 2-fold increase of specific enzyme activity (Table 10).

The impact of enzyme loading and carrier type was more moderately observable with Lipase PS preparates (Table 11).

Table 10 Kinetic resolution of 1-phenylethanol (*rac*-**1a**) with native, commercial sol-gel and with or without supported sol-gel Lipase AK with vinyl acetate as acylating agent. For sol-gel immobilization using binary sol-gel system (TEOS: PhTEOS=1:1) (TOMIN 2010c)

Biocatalysts	E/C ratio	*c* (%)	*E*[a]	U_B (µmol min^{-1}mg^{-1})	U_E (µmol min^{-1}mg^{-1})
Free Lipase AK[b]	-	49	»200	34.4	34.4
Without carrier[c]	-	51	>100	2.9	5.7
Celite	1/5	33	»200	1.9	12.2
Celite$_{pread}$	1/5	31	»200	1.8	12.2
Silica	1/5	40	>200	2.3	14.0
Silica$_{pread}$	1/5	49	»200	2.9	19.7
Without carrier[d]	-	38	»200	2.2	8.2
Celite	1/10	45	»200	2.6	34.6
Celite$_{pread}$	1/10	31	»200	1.8	24.2
Silica	1/10	37	»200	2.1	29.2
Silica$_{pread}$	1/10	41	>200	2.4	32.3

[a] If not stated otherwise, formation of the (*R*)-esters were preferred. Enantiomer selectivity (*E*) was calculated from *c* and $ee_{(R)\text{-}2}$(CHEN et al. 1982). Due to sensitivity to experimental errors, *E* values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

[b] Free Lipase AK, after 2 h reaction.

[c] Sol-gel immobilized Lipase AK without carrier (C). The applied enzyme amount corresponds with 1/5 enzyme/carrier ratio.

[d] Sol-gel immobilized Lipase AK without carrier (C). The applied enzyme amount corresponds with 1/10 enzyme/carrier ratio.

Table 11 Kinetic resolution of 1-phenylethanol (*rac*-**1a**) with native, commercial sol-gel and with or without supported sol-gel Lipase PS with vinyl acetate as acylating agent. For sol-gel immobilization using binary sol-gel system (TEOS: PhTEOS=1:1) **(**TOMIN 2010c**)**.

Biocatalyst	E/C ratio[b]	*c* (%)	*E*[a]	U_B (µmol min^{-1}mg^{-1})	U_E (µmol min^{-1}mg^{-1})
Free Lipase PS[b]	-	47	»200	32.5	32.5
Without carrier[c]	-	36	»200	2.1	5.4
Celite	1/5	36	»200	2.0	29.0
Celite$_{pread}$	1/5	15	»200	0.9	6.6
Silica	1/5	43	»200	2.5	20.2
Silica$_{pread}$	1/5	32	»200	1.8	12.1
Without carrier[d]	-	36	»200	2.0	10.8
Celite	1/10	14	>200	0.8	10.2
Celite$_{pread}$	1/10	13	>200	0.7	12.4
Silica	1/10	32	»200	1.8	27.8
Silica$_{pread}$	1/10	23	»200	1.3	19.4

[a] If not stated otherwise, formation of the (*R*)-esters were preferred. Enantiomer selectivity (*E*) was calculated from *c* and $ee_{(R)\text{-}2}$ (CHEN et al. 1982). Due to sensitivity to experimental errors, *E* values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

[b] Free Lipase PS, after 2 h reaction.

[c] Sol-gel immobilized Lipase PS without carrier (C). The applied enzyme amount corresponds with 1/5 enzyme/carrier ratio.

[d] Sol-gel immobilized Lipase PS without carrier (C). The applied enzyme amount corresponds with 1/10 enzyme/carrier ratio.

Table 12. Kinetic resolution of 1-phenylethanol (*rac*-**1a**) octan-2-ol (*rac*-**1d**) as members of multisubstarte "A" and "B" systems with native, commercial sol-gel and with or without supported sol-gel Lipase AK with vinyl acetate as acylating agent. In sol-gel immobilization of lipases using binary and ternary sol-gel systems. Specific enzyme (U_E) and biocatalyst activity (U_B) and enantiomer selectivity (E) of the free and immobilized lipases are shown.

		octan-2-ol				**1-phenylethanol**			
Biocatalyst	Silane ratio[b]	*c* (%)	*E*[a]	U_B (U/g)	U_E (U/g)	*c* (%)	*E*[a]	U_B (U/g)	U_E (U/g)
Free Lipase AK[b]	-	47.8	7.8	22.7	22.7	49.5	>200	31.6	31.6
Commercial SG	-	26.0	7.2	12.3	12.3	47.2	»200	30.1	30.1
Comm. on pumice	-	1.2	2.3	0.6	0.6	5.7	>100	3.6	3.6
Without carrier[c]	1:1	49.3	6.9	5.9	1.8	50.8	»200	16.2	4.9
Silica	1:1	55.9	5.3	2.2	0.2	53.1	»200	2.8	0.3
$Silica_{pread}$	1:1	63.5	5.4	2.5	0.3	51.0	»200	8.1	0.9
Without carrier[c]	1:0.7:0.3	48.6	6.4	1.9	0.5	35.7	»200	22.8	5.5
Silica	1:0.7:0.3	62.6	5.3	2.1	0.2	53.4	»200	2.8	0.3
$Silica_{pread}$	1:0.7:0.3	64.6	4.7	2.6	0.3	53.4	»200	2.8	0.3

[a] If not stated otherwise, formation of the (*R*)-esters were preferred. Enantiomer selectivity (E) was calculated from *c* and $ee_{(R)\text{-}2}$ (CHEN et al. 1982). Due to sensitivity to experimental errors, *E* values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.
[b] Free Lipase AK, after 2 h reaction.
[c] Sol-gel immobilized Lipase PS without carrier (C). The applied enzyme amount corresponds with 1/5 enzyme/carrier ratio.

Table 13. Kinetic resolution of 1-phenylethanol (*rac*-**1a**) octan-2-ol (*rac*-**1d**) as members of multisubstarte "A" and "B" systems with native, commercial sol-gel and with or without supported sol-gel Lipase PS with vinyl acetate as acylating agent. In sol-gel immobilization of lipases using binary and ternary sol-gel systems. Specific enzyme (U_E) and biocatalyst activity (U_B) and enantiomer selectivity (E) of the free and immobilized lipases are shown.

		octan-2-ol				**1-phenylethanol**			
Biocatalyst	Silane ratio[b]	*c* (%)	*E*[a]	U_B (U/g)	U_E (U/g)	*c* (%)	*E*[a]	U_B (U/g)	U_E (U/g)
Free Lipase PS[b]	-	7.0	7.1	3.3	3.3	20.7	»200	13.2	13.2
Commercial SG	-	8.3	9.5	3.9	3.9	24.0	»200	15.3	15.3
Without carrier[c]	-	12.1	9.2	5.8	0.8	31.3	»200	20.0	2.8
Silica	1:1	14.0	8.3	3.3	0.4	36.7	»200	11.7	1.3
$Silica_{pread}$	1:1	11.5	9.6	5.5	0.5	31.4	»200	20.1	1.8
Without carrier[c]	1:0.7:0.3	29.1	11.9	13.8	4.3	51.0	»200	32.5	10.1
Silica	1:0.7:0.3	17.6	7.8	4.2	0.5	40.5	»200	12.9	1.6
$Silica_{pread}$	1:0.7:0.3	12.6	8.6	1.5	0.2	31.7	»200	5.1	0.8

[a] If not stated otherwise, formation of the (*R*)-esters were preferred. Enantiomer selectivity (E) was calculated from *c* and $ee_{(R)\text{-}2}$ (CHEN et al. 1982). Due to sensitivity to experimental errors, *E* values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.
[b] Free Lipase PS, after 2 h reaction.
[c] Sol-gel immobilized Lipase PS without carrier (C). The applied enzyme amout corresponds with 1/5 enzyme/carrier ratio.

Generally it can be stated that for future experiments silica (Geduran® Si60) is preferred without preadsorption for lipase immobilization in between 1:4 and 1:10 carrier: enzyme ratio.

Villeneuve and co-workers (2000) published that immobilization can sometimes protect lipase from inactivation by chemicals produced during the enzyme-catalyzed reaction. The inactivating effect of acetaldehyde toward some lipases has been established several cases. This co-product is formed when vinyl esters are used as irreversible acyl donors in esterification reactions. Observation that inactivation of *Candida rugosa* lipase by acetaldehyde can be prevented by immobilization of the enzyme was confirmed by our results.

Table 14. Kinetic resolution of 1-phenylethanol (*rac*-**1a**) octan-2-ol (*rac*-**1d**) as members of multisubstarte "A" and "B" systems with native, commercial sol-gel and with or without supported sol-gel Lipase CrL with vinyl acetate as acylating agent. In sol-gel immobilization of lipases using binary and ternary sol-gel systems. Specific enzyme (U_E) and biocatalyst activity (U_B) and enantiomer selectivity (E) of the free and immobilized lipases are shown.

		octan-2-ol				**1-phenylethanol**			
Biocatalyst	Silane ratio[b]	*c* (%)	*E*[a]	U_B (U/g)	U_E (U/g)	*c* (%)	*E*[a]	U_B (U/g)	U_E (U/g)
Free Lipase CrL[b]	-	11.5	2.0	5.5	5.5	5.0	2.3	3.2	3.2
Commercial SG	-	0.4	1.7	0.2	0.2	0.2	1.6	0.1	0.1
Without carrier[c]	1:1	10.7	1.8	0.1	0.1	2.7	3.8	0.4	0.1
Silica	1:1	6.8	3.4	0.8	0.1	11.3	9.9	3.6	0.3
$Silica_{pread}$	1:1	6.1	0.8	0.2	0.02	6.5	9.4	0.3	0.03
Without carrier[c]	1:0.7:0.3	4.6	1.5	0.2	0.05	4.7	4.4	0.2	0.1
Silica	1:0.7:0.3	5.2	2.7	0.2	0.02	10.6	38.0	0.6	0.1
$Silica_{pread}$	1:0.7:0.3	11.8	3.9	0.5	0.06	7.6	10.8	2.4	0.3

[a] If not stated otherwise, formation of the (*R*)-esters were preferred. Enantiomer selectivity (*E*) was calculated from *c* and $ee_{(R)\text{-}2}$ (CHEN et al. 1982). Due to sensitivity to experimental errors, *E* values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

[b] Free Lipase CrL, after 2 h reaction.

[c] Sol-gel immobilized Lipase PS without carrier (C). The applied enzyme amount corresponds with 1/5 enzyme/carrier ratio.

5.2.5.2. Stability

The sol-gel entrapped lipases are highly stable and can be stored at room temperature for months without significant loss of activity. For instance as literature data show (REETZ et al. 1995) immobilized lipase from *Pseudomonas cepacia* in MTMS/PDMS (methyltrimethoxysilane, polydimethylsiloxane) gels has been repeatedly used in batch esterification reaction of lauric acid with 1- octanol in isooctane.

We also performed a heat stability test from 40 °C upto 80 °C with sol-gel immobilized Lipase PS in ternary silane precursor system (TEOS: OcTEOS:PheTEOS) showed very promising results. The biocatalyst was refluxed for one hour at five different temperatures (40, 50, 60, 70, 80 °C) in octanol.Then it was filtered and tested in kinetic resolution reactions of multisubstrate "A" and "B" using vinyl acetate as acylating agent. The results showed that our sol-gel biocatalyst even at 80°C retained at least ~50% its activity.

A storage test was also performed at ambient temperature for half year our sol-gel immobilized Lipase PS preadsorbed on silica kept its initial activity.

5.3. Bioimprinting

Bioimprinting effect in sol-gel immobilization of lipases was studied to develop efficient novel immobilized biocatalysts with significantly improved properties for biotransformations in continuous-flow systems. The substrate mimics within the active site in experimental lipase structures (PDB) or structurally related compounds were selected systematically as bioimprinting candidates. Four lipases (from Lipase AK, Lipase PS, lipase B from *Candida antarctica* and lipase *Candida rugosa*) were immobilized by sol-gel process with nine bioimprinting candidates using various combinations of tetraethoxysilane (TEOS), phenyltriethoxysilane (PhTEOS), octyltriethoxysilane (OcTEOS) and dimethyldiethylsilane (DMDEOS) as silica precursors. The biocatalytic properties of the immobilized lipases were characterized by enantiomer selective acylation of various racemic secondary alcohols in multisubstrate systems (tests with a two component system: *rac*-**1a**,**b** and a five component system: *rac*-**1c-g**). The biocatalytic usefulness of the best preparations was demonstrated by the kinetic resolution of racemic 1-(thiophen-2-yl)ethanol (*rac*-**1h**) in batch and continuous-flow systems.

The sol-gel entrapment can „freeze" the conformation of the enzyme; it is thus understandable that the pre-immobilization conformation of the lipase is very important. Furthermore the conditions selected should be sufficient to result in a more stable conformation on the enzyme molecule. If the lid can be kept open, lipase is expected to have a high activity in organic solvents (HELLNER et al. 2011).

Based on these bioimprinting was investigated in the following section.

5.3.1. Rational selection of the imprinting molecules for lipases

In 1990, the first 3D structures of lipases from *Rhizomucor miehei* (BRADY et al.) and human pancreatic lipase (WINKLER et al.) were elucidated. In 1993, the interfacial activation of the lipase from *Rhizomucor miehei* was rationalized on structural basis with substrate like inhibitors (DEREWENDA et al. 1992). Since then 121 lipase structures have been deposited in the Protein Data Bank (Brookhaven PDB). Among the 76 lipase structures containing ligands, those structures which included non-covalently bound ligands mimicking the substrates were selected (Figure 22).

The non-covalently bound ligands mimicking the arrangement of substrates in the experimental structures can be divided into three categories. As it was expected, free fatty acids were found within lipases in the close proximity of the catalytically active Ser (stearic acid in Figure 22 A and oleic acid in Figure 22 B). Not surprisingly, non-ionic surfactants occupying the active site were also found in lipases (hydroxy-ethyloxy-triethyloxy-octane in Figure 22 C and methylpenta(oxyethyl) heptadecanoate in Figure 22 D). Interestingly, only the polyethyleneglycol part of the non-ionic surfactant Triton X-100 is visible in the structure of a thermoalkalophilic

lipase (Figure 22 E) and only the pentaethylene-glycol was also found as substrate mimic in the structure of lipase A from *Candida antarctica* (Figure 22 F).

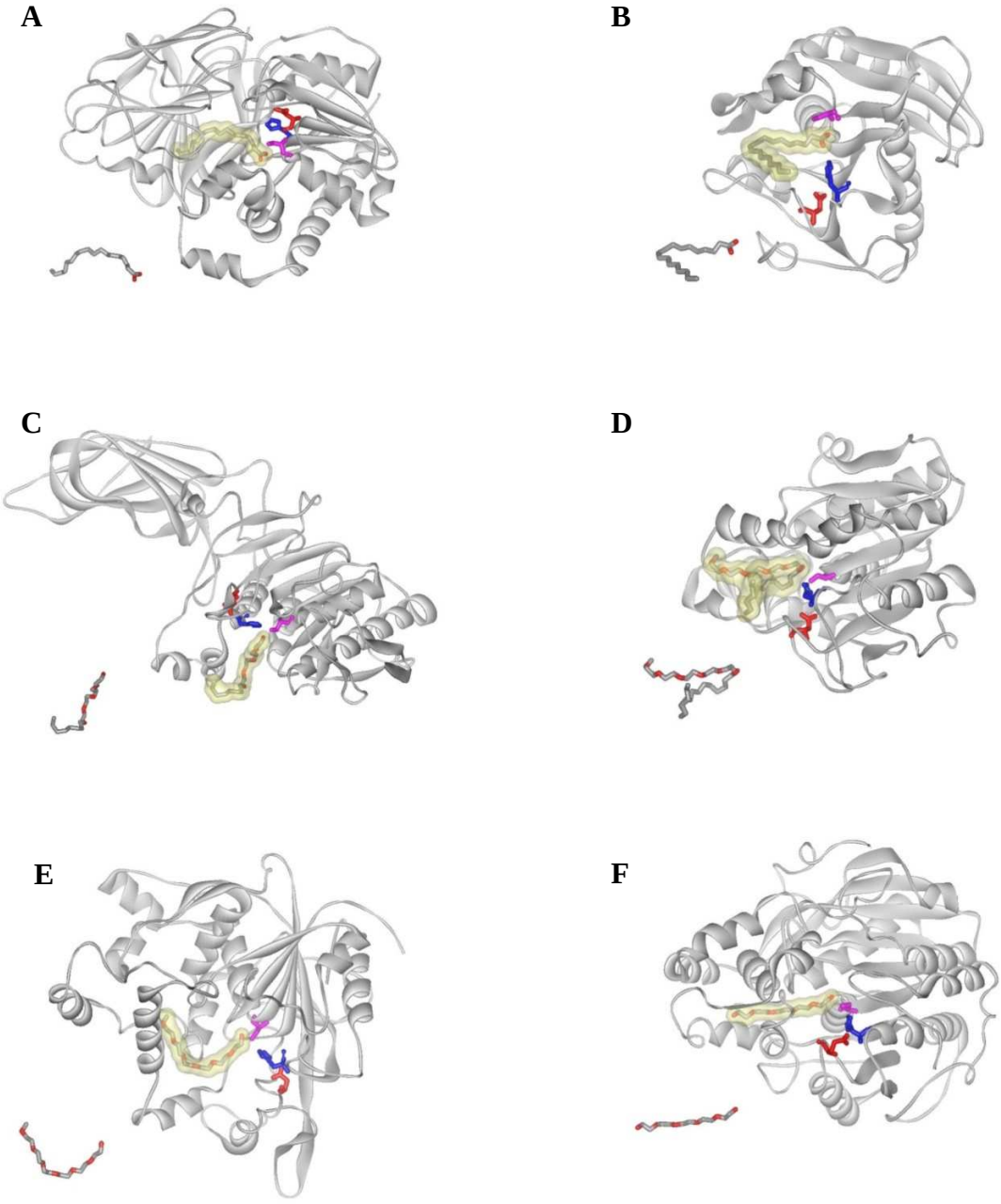
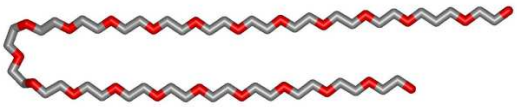
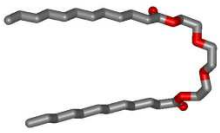
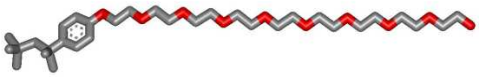
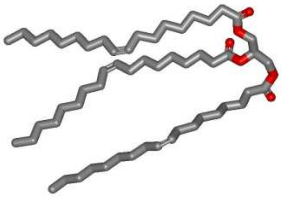


Figure 22. Crystal structures of lipases including substrate analogues within the active site **A)-F)** For better visibility, the catalytic triad is colored (Ser: magenta, His: blue, Asp/Glu: orange) and the amphiphile present in the active site is repeated in the left down corner for better visibility; **A)** *Fulgidus* sp. lipase with stearic acid (PDB code: 2ZYI) (CHEN et al. 2009); **B)** *Thermomyces lanuginosa* lipase with oleic acid (PDB code: 1GT6) (YAPOUDJIAN et al. 2002) **C)** Porcine pancreatic lipase - colipase with hydroxy-ethyloxy-triethyloxy-octane (PDB code: 1ETH); (HERMOSO et al. 1996) **D)** *Candida antarctica* lipase B with methylpenta(oxyethyl) heptadecanoate (PDB code: 1LBT) (UPPENBERG et al. 1995); **E)** Bacterial thermoalkalophilic lipase with Triton X-100 (PDB code: 2W22) (CARRASCO-LOPEZ et al. 2009); **F)** *Candida antarctica* lipase A with pentaethylene glycol (PDB code: 3GUU) (BRANDT et al.).

Because our analysis of experimental lipase structures indicated various types of molecules as substrate mimics, it was expected that the best imprinting molecule may vary from one lipase to another. Therefore, nine imprinting candidates (Table 15) have been selected for this systematic study including substrate [olive oil (OA)], products [lauric acid (LA) and oleic acid (OA)], non-ionic surfactants [polyethylene glycol dodecyl ether (BRIJ-30) and polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100)] and polyethylene glycol derivatives [tetraethylene glycol (TEG), polyethylene glycol 400 (PEG 400) and 1000 (PEG 1000), and dilaurate of tetraethylene glycol (L-TEG-L)].

Table 15. Structure of imprinting molecules selected for sol-gel entrapment of lipases (red color means oxygen molecule)

Additives	Name	Structure
LA	Lauric acid	
OA	Oleic acid	
TEG	Tetraethylene glycol	
PEG400	Polyethylene glycol 400	
PEG1000	Polyethylene glycol 1000	
BRIJ	BRIJ-30 (polyethylene glycol dodecyl ether)	
L-TEG-L	Tetraethylene glycol dilaurate	
TRX100	Triton X-100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether)	
Olive oil	Triolein	

The imprinting properties of the rationally selected additives were tested on four different lipases of well documented properties to test the generality of our approach. Lipases from diverse types of microorganisms such as CaLB and CrL from yeasts and Lipase AK and Lipase PS from bacterial source were selected. Our selection was also diverse by the conformational mobility of the lipases. Whereas CrL is the more sensitive to conformation changes, Lipase AK and Lipase PS and especially CaLB and are more heat resistant and respectively less mobile. The most dramatic imprinting effects were expected in case of the conformation sensitive lipases.

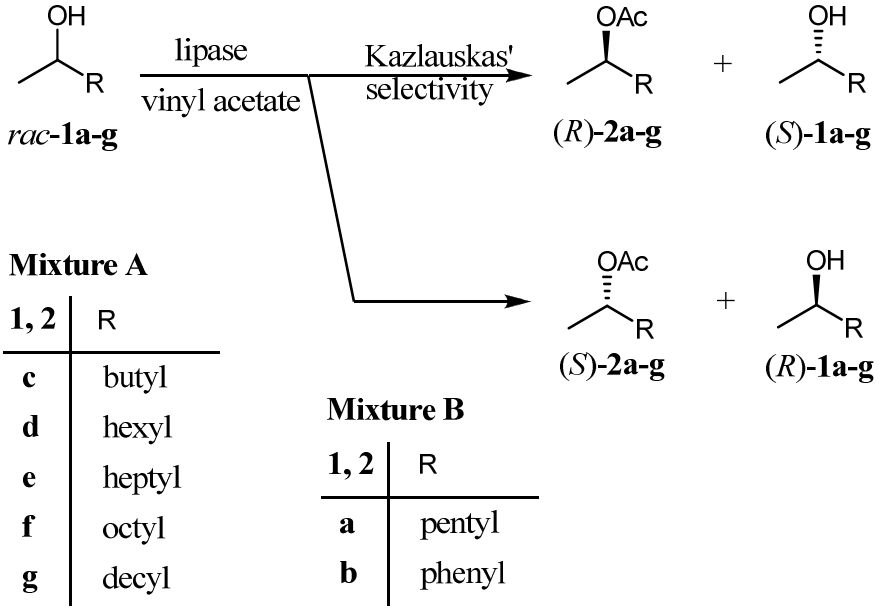
It was expected also, that encapsulation of the lipases can "freeze" their conformation and therefore an encapsulated lipase can "remember" the imprinting effect even after removal of the template molecule. Studies on subtilisin revealed that this behavior (ligand induced memory) is a result of the decreased flexibility displayed by enzymes in an apolar solvent, relatively to the aqueous situation (RUSSELL and KLIBANOV1988, LOUSA et al. 2011). Because the mobility of the enzyme entrapped in rigid sol-gel matrix is extremely low, it is understandable that selection of suitable imprinting additives might be crucial to improve the activity of the immobilized enzyme.

In a comprehensive study on sol-gel immobilization of lipases, enhancing effects of various small molecules (Tween 80, 18-crown-6 and methyl-β-cyclodextrin) and supporting materials such as Celit were investigated simultaneously (REETZ et al. 2003). However, the enhancing effects in sol-gel immobilization systems by small molecules and solid supports may be different. The enhancing effect in supported sol-gel techniques is mainly due to formation of a thin enzyme layer on the large surface of the supporting material lowering the diffusion limits which are present in enzyme aggregates. On the other hand, in case of small molecules as additives induced conformational changes of the proteins can mostly contribute to the enhancement of enzyme activity. Although the supported sol-gel systems proved to be quite efficient in our previous studies as well, (TOMIN et al. 2011) this study was designed in non-supported sol-gel systems to detect solely the imprinting effect. To further simplify the investigated sol-gel system, the most effective binary precursor system [TEOS: PhTEOS (1:1)] of our previous work (TOMIN et al. 2011) was selected to study the imprinting effect of the selected additives.

5.3.2. Multisubstrate kinetic resolution as test system for the preliminary investigations

In order to distinguish the effects due to induced conformation change of the enzyme and due to the various hydrophobicity of encapsulating matrices, tests of the imprinted biocatalysts were designed with multisubstrate mixtures consisting of a series of racemic aliphatic alcohols of various chain lengths (Figure 23). Another mixture was applied to compare the behavior of an aliphatic and aromatic secondary alcohol directly. Thereafter the two substrate mixtures were tested with two

additional ternary sol-gel systems of different hydrophobicity. These two ternary systems were the ones which proved to be the most effective in our two previous studies (TOMIN et al. 2011), (WEISER et al. 2011). It was considered that conformation-controlled effect can cause less variation of the enzyme activity within the members of the homologous series than matrix hydrophobicity dependent effects. If an effect is mainly due to matrix hydrophobicity, the relative activity between substrates of different hydrophobicity should be more pronounced.



Mixture A

1, 2	R
c	butyl
d	hexyl
e	heptyl
f	octyl
g	decyl

Mixture B

1, 2	R
a	pentyl
b	phenyl

Figure 23. Multiple kinetic resolutions with substrate mixtures for testing the various biocatalysts

Because the lipases may be sensitive to variations in the nature and area of the forming aqueous-organic interface, the sol-formation steps (mixing the silane precursors with the aqueous phase, time of the addition the enzyme and the NaF initiator, omitting or applying ultrasonication) were varied with two enzymes (Lipase PS and CrL) in a series of preliminary experiments. In case of CrL the intensive emulsion formation was crucial and thus ultrasonication had beneficial effect on the activity of the resulting biocatalyst. On the other hand, variations in the sequence of sol-formation resulted in no significant alterations in the properties of the immobilized biocatalysts from Lipase PS.

Next, initial experiments were carried out to investigate the effect of the amount of additive in the sol-gel formation on the properties of the encapsulated lipases. In this case imprinting Lipase PS and CrL with PEG400 as additive were selected for this study (Figure 24).

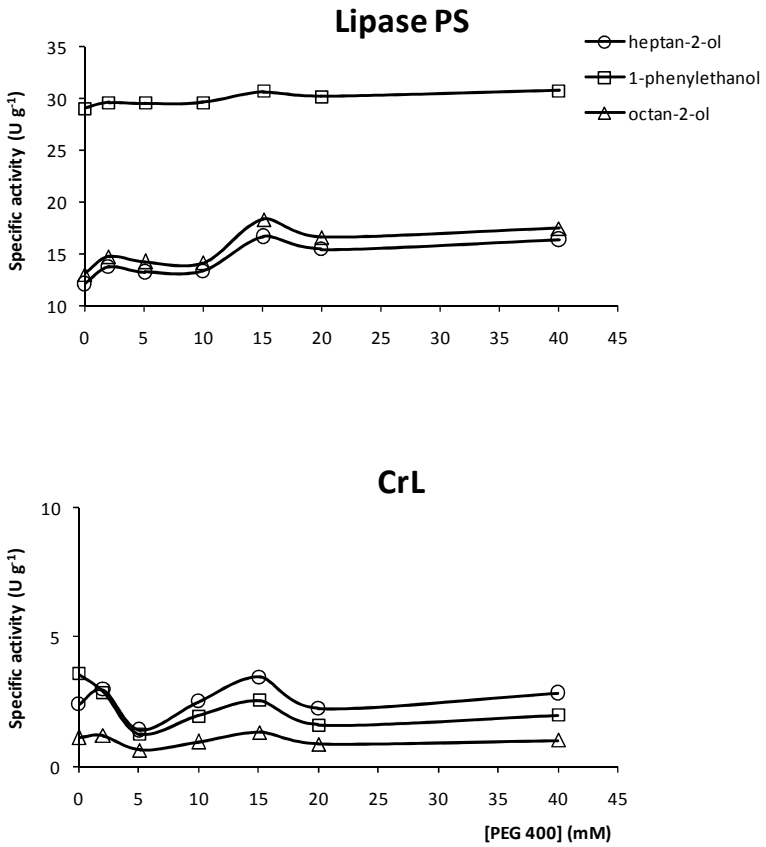


Figure 24. Dependence of the specific activity (U_B, U g^{-1}) on the concentration of PEG 400 as imprinting additive for Lipase PS (2h) and CrL (8h) (for details see Annex 3)

Kinetic studies on various lipases with various substrates demonstrated that the Michaelis constants fall in the 0.1-40 mM range, depending on the nature of the lipase and substrate (K_m= 2.2 mM and K_m= 5.1 for lipase from porcine pancreas with sunflower oil and palm oil, respectively; (ARROYO et al. 1996) K_m= 0.15 mM for lipase from *Pseudomonas aeruginosa* on *p*-nitrophenyl palmitate (MADAN and MISHRA 2010); K_m= 38 mM for lipase A from *Candida antarctica* with *p*-nitrophenyl butyrate (HE et al. 2010); K_m= 0.22 mM for pancreatic lipase on *p*-nitrophenyl acetate. (WONG 1995, WHITAKER et al. 2003) Because the concentration of the PEG 400 additive is comparable with the K_m-values of the substrates, it may be hypothesized that the first increase observable with both enzymes is related with the substrate analogue-like behavior. The second increase in the specific activity of the entrapped lipases (K_m= 5-20 mM interval) can be the mutual effects of the substrate analogue-like habit and the impact of the additive on the quality of matrix. It is worthwhile to mention here that similar two stages increase has been observed for porcine pancreatic phospholipase A_2 imprinted by octyl-β-D-glucopyranoside (MINGARRO et al. 1995).

In this work, the second observed increase was attributed to the appearance of interfaces, and activity increase was also saturable at higher concentrations. Based on these considerations, the

further investigations have been performed at 10-20 mM concentration of the designed imprinting molecules.

5.3.3. Imprinting effect of the selected additives on various lipases

To evaluate the imprinting effects in sol-gel immobilization of lipases, multisubstrate test reactions were used and the resulting specific activities (U_B), activity yields (Y_A) and enantiomer selectivities (E) were compared (Table 16). Worth noting that our sol-gel entrapped biocatalysts even without imprinting additives were superior to the corresponding commercially available sol-gel lipases [SG (-) vs. SG comm. preparations from Lipase AK, Lipase PS and CrL in Table 16)]

Table 16. Screening the imprinting effect of the additives in sol-gel immobilization of lipases using binary sol-gel system (TEOS: PhTEOS=1:1). Specific activity (U_B) and enantiomer selectivity (E) of the free and immobilized lipases are shown with octan-2-ol *rac*-**1d** (Panel A) and with 1-phenylethanol *rac*-**1a** (Panel B). Reaction times were 2 h for Lipase AK, Lipase PS and CaLB and 8 h for CrL (for further reaction details, see Methods section and Annex 4-11).

Form (Additive)[a]	**Lipase AK**		**Lipase PS**		**CaLB**		**CrL**	
	U_B (U g^{-1})	E[b]	U_B (U g^{-1})	E[b]	U_B (U g^{-1})	E[b]	U_B (U g^{-1})	E[b]
Panel A (*rac*-**1d**)								
commercial[c]	23	7.8	3	7.1	48	>100	2.2	1.9[d]
SG comm.[e]	13	7.2	4	9.5	24[e]	96[e]	0.1	2.1[d]
SG (-)	23	6.2	23	10	9	»200	0.7	1.8[d]
SG (LA)	12	4.0	25	10	11	>200	0.4	2.2[d]
SG (OA)	20	5.1	21	9.5	15	>200	0.7	2.0[d]
SG (TEG)	17	4.6	20	9.5	10	>200	0.4	1.9[d]
SG (PEG400)	19	5.0	23	11	12	>200	0.5	1.8[d]
SG (PEG1000)	20	5.2	19	9.2	16	>200	0.5	2.0[d]
SG (BRIJ)	11	4.2	11	5.7	22	»200	2.3	1.7[d]
SG (L-TEG-L)	5	2.1	24	10	17	>200	0.3	1.9[d]
SG (TRX100)	17	4.4	6	5.7	21	>200	2.4	1.6[d]
SG (Olive oil)	15	4.3	11	6.4	18	>200	0.5	1.8[d]
Panel B (*rac*-**1a**)								
commercial[c]	31	>200	13	>200	126	»200	1.6	2.4
SG comm.[e]	30	»200	15	»200	31[e]	»200[e]	0.05	2.5
SG (-)	31	>200	31	»200	16	>200	0.7	3.7
SG (LA)	30	>100	32	»200	14	»200	0.2	3.8
SG (OA)	26	>200	30	»200	19	»200	0.5	3.6
SG (TEG)	26	>100	31	»200	8	>200	0.3	3.9
SG (PEG400)	32	>200	31	»200	20	>200	0.5	3.1
SG (PEG1000)	29	>100	31	»200	13	>200	0.5	3.1
SG (BRIJ)	30	>100	25	>200	25	»200	2.2	3.5
SG (L-TEG-L)	29	>100	31	»200	10	>200	0.3	3.5
SG (TRX100)	30	>100	17	>200	17	»200	2.4	3.2
SG (Olive oil)	14	>100	26	»200	15	»200	0.5	3.7

[a] Sol-gel immobilized enzyme without or with additive (0.5 v/v%).

[b] If not stated otherwise, formation of the (*R*)-esters were preferred. Enantiomer selectivity (E) was calculated from c and $ee_{(R)\text{-}2}$ (CHEN et al. 1982). Due to sensitivity to experimental errors, E values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

[c] The corresponding commercially available native lipase powder for Lipase AK, Lipase PS and CrL. For CaLB, the commercial liquid enzyme (Novozym CALB L) was immobilized on Novozym carrier (1 mg carrier to 8 µl enzyme solution).

[d] The formation of (*S*)-**2d** is preferred.

[e] The corresponding commercial sol-gel immobilized enzyme for Lipase AK, Lipase PS and CrL. For CaLB, Novozym 435 was used for comparison.

Because the activities in the present work were calculated from single time-point data, the term "activity" must be considered only for comparative evaluation of the catalytic efficiencies, not for the real kinetic behavior of the biocatalyst. In this comparison, the effective specific activities (U_B) and enantiomer selectivities (E) of the immobilized biocatalysts are included [further data such as activity yields (Y_A) (TOMIN et al. 2011) are in the Appendix].

In the case of Lipase AK, the sol-gel entrapment resulted in robust immobilized biocatalysts with the same effective specific activity as the free lipase powder. This represents more than 2.2-fold increase in the activity of Lipase AK upon immobilization (Y_A= 224 %, in sol-gel matrix without additive). Whereas the sol-gel immobilization of Lipase AK led to such a large activity enhancement, none of the imprinting additives could achieve further improvement. This result can be explained in two ways. One explanation may be that Lipase AK is not sensitive to imprinting. Another explanation – which we feel more plausible – may be that phenyltriethoxysilane or its partially hydrolyzed derivatives play the same role (Figure 25 B) as the imprinting additives (Figure 24. A). This assumption is supported by the fact that lipases can accept alkoxysilanes as substrates as indicated by the study on a lipid coated lipase which was able to catalyze the oligomerization of dimethyldiethoxysilane. (NISHINO et al. 2001) Accordingly, phenyltriethoxysilane at high concentration can occupy the active site of the lipase (Figure 25 B) and additives at lower concentration could not achieve further imprinting effect on this enzyme.

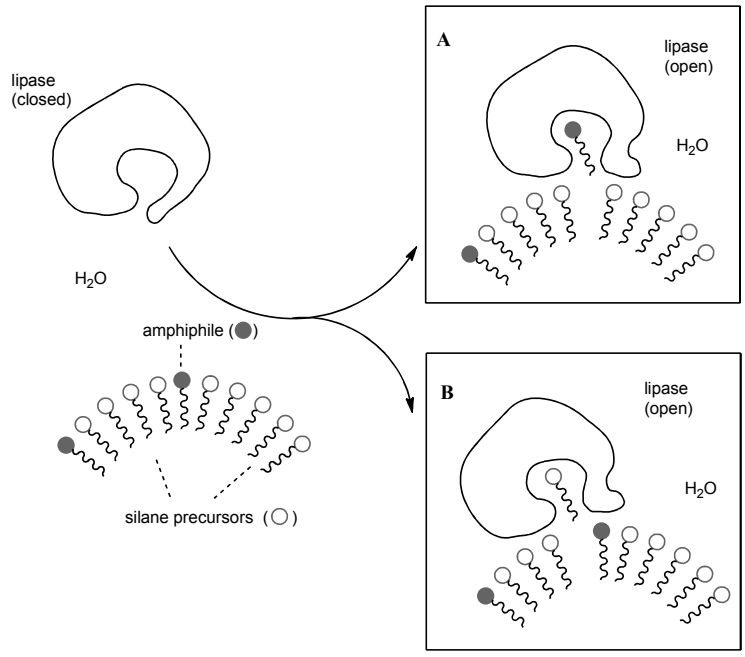


Figure 25. Illustration of interfacial activation based molecular imprinting of lipase with amphiphile (A) or with silane precursors (B) (HELLNER et al. 2011)

The sol-gel entrapment resulted in quite efficient immobilized Lipase PS biocatalysts with remarkably higher effective specific activity as the free lipase powder (U_B= 31 U g^{-1} vs. 13 U g^{-1} for SG (-) and the Lipase PS powder, respectively, with *rac*-**1a** as substrate). This remarkable efficiency increase is even more pronounced when the activity yields are taken into account [Y_A ~2500 % for SG (-) with *rac*-**1d** as substrate, see Supplementary Information]. Similarly to the case of Lipase AK, it might be supposed that phenyltriethoxysilane or its derivatives possess imprinting effect on Lipase PS during the sol-gel entrapment process. However, lauric acid as additive exhibited a slight additional imprinting effect with Lipase PS [e.g. U_B= 23 U g^{-1} vs. 25 U g^{-1} for SG (-) and SG (LA), respectively, with *rac*-**1d** as substrate]. It is in agreement with the previous results on the lipase from *Burkholderia cepacia* in a sol-gel system from methyltrimethoxysilane (MTMOS) and tetramethoxysilane (TMOS) precursors, yielding a bioimprinted lipase with 47.9- and 2.5-fold increase in specific activity over the free and non-imprinted immobilized lipases, respectively (CAO et al. 2009).

In the sol-gel entrapment of lipases of yeast origin (CaLB and CrL), BRIJ and TRX100 had remarkable imprinting effect on the specific activity of the immobilized enzymes [e.g. U_B= 9 U g^{-1} vs. 22 U g^{-1} for SG (-) and SG (BRIJ), respectively, for CaLB with *rac*-**1d** as substrate; or U_B= 0.7 U g^{-1} vs. 2.4 U g^{-1} for SG (-) and SG (TRX100), respectively, for CrL with *rac*-**1d** as substrate; in Table 17)]. Not surprisingly, the polyethylene glycol ether structures of BRIJ 30 and Triton X-100 are highly reminiscent of the imprinting molecules found in the related experimental lipase structures (Figure 22 C,D,F). Interestingly, not only the specific activity but the enantiomer selectivity of the sol-gel entrapped Lipase PS, CaLB and CrL was enhanced also to some extent. Such enhancement in enantiomer selectivity can be rationalized by supposing that the new conformations adopted by these lipases in the presence of the efficient imprinting molecules might be even more favoring for the faster reacting enantiomers.

It is worthwhile to note that CrL preferred the acylation of (*S*)-enantiomers of the aliphatic secondary alcohols *rac*-**1a-f**. This is not surprising, because earlier it was found that coating of CrL with surfactant molecules affects the recognition of an alcohol substrate and changes the stereopreference (KAMIYA and GOTO 1998).

5.3.4. Scaling up the imprinted sol-gel lipases for continuous-flow applications

Lipases can be applied advantageously in continuous systems (PATEL et al. 1996, CHEN and TSAI 2000, UJANG et al. 2003, LIESE et al.2006, CSAJÁGI et al. 2008, TOMIN et al. 2010a). Although the free native lipases behave as enzyme powders in organic media and can be used as fillings in packed-bed bioreactors (CSAJÁGI et al. 2008) one problem to overcome is the relatively low activity and stability of the non-immobilized enzyme aggregates. High lipase input obviously reduces the economic viability of the system and hence less feasible. Therefore, a packed-bed reactor filled with robust sol-gel immobilized enzyme is an obvious alternative.

A recent study confirmed that lipases entrapped in sol-gel matrices from ternary silane precursor systems surpassed the catalytic properties of corresponding immobilized biocatalysts from binary silane precursor systems (TOMIN et al. 2011). It was also found that lipases in ternary sol-gel systems with dimethyldiethoxisilane (DMDEOS) as one of the silane precursors had even more beneficial properties (WEISER et al. 2011). Therefore, based on the imprinting effects of various additives with the investigated four lipases in a binary sol-gel system, the best candidates resulting in the most selective and most productive biocatalysts were investigated further in ternary sol-gel systems. Our further goal was to compare the properties of the best combinations of imprinted lipases in ternary sol-gel systems as biocatalysts in bath and in continuous-flow mode kinetic resolutions with multiple substrates and with a single substrate on preparative scale.

First, scaling up the immobilization of the lipases with the best performing additives in binary sol-gel matrices from sub-gram scale to multi-gram scale was performed and extended to the best combinations of the ternary compositions [TEOS:OcTEOS:PhTEOS 10:7:3 (TOMIN et al. 2011) and TEOS:PhTEOS:DMDEOS 4:1:1 (WEISER et al. 2011)] found earlier (Table 17).

Table 17. Comparison of lipases imprinted with the best performing additives as immobilized biocatalysts in binary and ternary sol-gel matrices. Specific activity (U_B), activity yield (Y_A) and enantiomer selectivity (E) of the free and immobilized forms of lipases in multiple kinetic resolutions are shown in Panel A (for octan-2-ol *rac*-**1d**) and Panel B (for 1-phenylethanol *rac*-**1a**). The reaction times were 0.5 h for Lipase AK, Lipase PS and CaLB and 4 h for CrL in hexane:THF 2:1 solvent (for further reaction details, see Materials and Methods section and Annex 12-15).

Silane precursors (enzyme amount)	**Lipase AK**[a]			**Lipase PS**[a]			**CaLB**[a]			**CrL**[a]		
	U_B (U g^{-1})	Y_A (%)	E[b]	U_B (U g^{-1})	Y_A (%)	E[b]	U_B (U g^{-1})	Y_A (%)	E[b]	U_B (U g^{-1})	Y_A (%)	E[b]
Panel A (*rac*-**1d**)												
-[c]	34	100	5.2	4	100	4.3	186	100	>200	10.5	100	2.3[d]
TEOS:PhTEOS 1:1 (1×)[e]	42	274	4.3	30	1854	7.1	63	2571	»200	2.8	37	2.0[d]
TEOS:OcTEOS:PhTEOS 10:7:3 (1×)[e]	111	835	9.4	54	3741	6.7	85	2829	>200	0.4	43	1.5[d]
TEOS:OcTEOS:PhTEOS 10:7:3 (2×)[f]	118	409	12.0	120	3760	11.0	92	1459	»200	0.9	49	1.8[d]
TEOS:PhTEOS:DMDEOS 4:1:1 (1×)[e]	12	145	5.8	59	6063	8.3	79	4611	>200	0.6	481	1.3[d]
TEOS:PhTEOS:DMDEOS 4:1:1 (2×)[f]	58	327	5.6	101	5222	11.0	91	2095	>200	1.1	119	1.7[d]
Panel B (*rac*-**1a**)												
-[c]	109	100	>200	23	100	>200	507	100	»200	4.4	100	2.3
TEOS:PhTEOS 1:1 (1×)[e]	98	198	>200	94	1080	»200	86	1150	»200	2.3	75	3.1
TEOS:OcTEOS:PhTEOS 10:7:3 (1×)[e]	127	295	>200	118	1520	»200	76	843	>200	0.1	14	5.3
TEOS:OcTEOS:PhTEOS 10:7:3 (2×)[f]	127	136	>200	128	737	>200	125	652	»200	0.8	165	4.6
TEOS:PhTEOS:DMDEOS 4:1:1 (1×)[e]	74	283	>200	125	2363	»200	117	2263	»200	0.8	681	9.2
TEOS:PhTEOS:DMDEOS 4:1:1 (2×)[f]	126	217	»200	126	1210	»200	113	857	»200	1.2	95	6.1

[a] Imprinting additives: no additive for Lipase AK, LA (0.5 v/v%) for Lipase PS, BRIJ (0.5 v/v%) for CrL, BRIJ for (0.5 v/v%) for CaLB.

[b] If not stated otherwise, formation of the (*R*)-esters were preferred. Enantiomer selectivity (E) was calculated from c and $ee_{(R)\text{-}\mathbf{2}}$ (CHEN et al. 1982) Due to sensitivity to experimental errors, E values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

[c] The corresponding commercially available native lipase powder for Lipase AK, Lipase PS and CrL. For CaLB, the commercial liquid enzyme (Novozym CALB L) was immobilized on Novozym carrier (1 mg carrier to 8 µl enzyme solution).

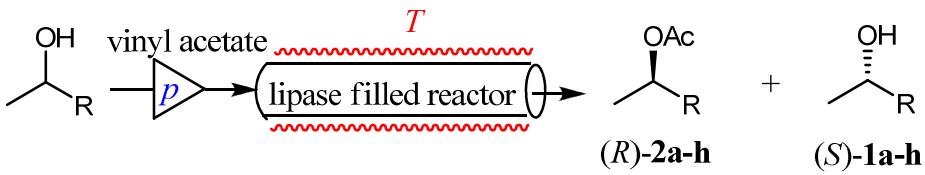
[d] The formation of (*S*)-**2d** is preferred.

[e] Normal (single) enzyme loading (for further details, see Experimental Section).

[f] Double enzyme loading (for further details, see Experimental Section).

Expectedly, the lipases performed better in the ternary sol-gel systems than in the binary TEOS:PhTEOS 1:1 systems using the additive which proved to be the most effective in the preliminary tests (Table 17: no additive for Lipase AK, LA for Lipase PS, BRIJ for CrL and CaLB). Although the doubled amount of the entrapped lipases usually resulted in higher specific activity (U_B) of the forming sol-gel biocatalysts, this activity enhancement increased not proportionally with the enzyme loading. In all cases, either of the ternary sol-gel systems with the lower lipase loading resulted in the higher activity yield (Y_A). Regarding the specific activity (U_B) and enantiomer selectivity (E) together, the most efficient systems were obtained from the TEOS:PhTEOS:DMDEOS 4:1:1 silane precursors with high lipase loading. It is also obvious, that the microenvironment of the resulting matrix has to be taken into consideration. Correlation was found between the order of specific activity (U_B) for the various members of the homologous series *rac*-**1c-g** and the hydrophobicity of the silane precursors (see Appendix).

After selecting the lipases with high lipase loading in TEOS:PhTEOS:DMDEOS 4:1:1 silane precursor systems containing the best imprinting molecules (no additive for Lipase AK, LA for Lipase PS, BRIJ for CrL and CaLB), the productivity (r) and enantiomer selectivity (E) in kinetic resolutions with the sol-gel lipases were compared in batch mode and in continuous-flow reactor. The tests were performed with the multisubstrate systems (Mixture A and B in Figure 26) and with the single racemate *rac*-**1h** (Figure 26). The solvent system for this series of investigation was hexane: TBME: vinyl acetate 6:3:1, which is more compatible with the usual pump sealings than the systems consisting of THF.



Mixture A

1, 2	R
c	butyl
d	hexyl
e	heptyl
f	octyl
g	decyl

Mixture B

1, 2	R
a	pentyl
b	phenyl

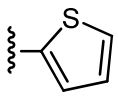
1, 2	R
h	

Figure 26. Multiple substrate (*rac*-**1a,b** and *rac*-**1c-g**) and single substrate (*rac*-**1h**) kinetic resolutions in continuous-flow reactors. [▷: pump; ~~: temperature control unit].

The comparison of batch mode and continuous-flow mode with the multisubstrate systems for the four selected sol-gel lipases preparations revealed that independently of the substrate (*rac*-**1a-g**) or enzyme (Lipase AK, Lipase Ps, CaLB or CrL), the productivity (specific reaction rate, *r*) of the continuous-flow system always exceeded the corresponding value for the batch mode with the same enzyme preparation (Figure 27). These results were in agreement with our previous data obtained for kinetic resolutions of single racemates with immobilized lipases in continuous-flow reactors (CSAJÁGI et al. 2008 and TOMIN et al. 2010a).

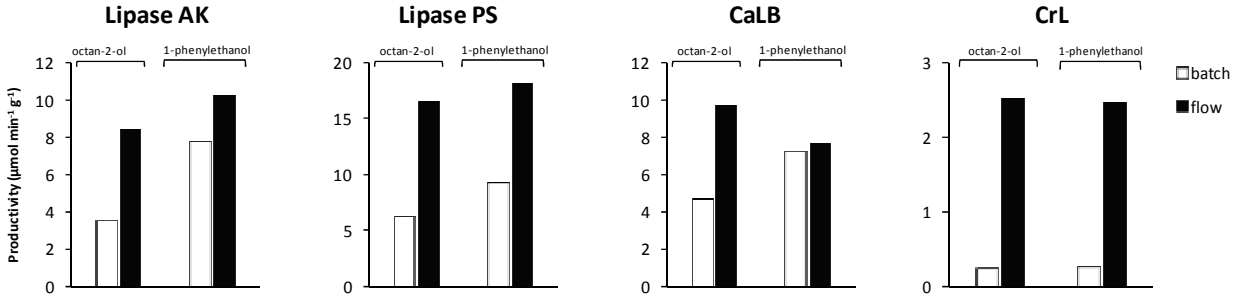


Figure 27. Comparison of the productivity (specific reaction rate, *r*) for the best performing imprinted sol-gel lipases in batch mode and in continuous-flow packed-bed reactor using multiple kinetic resolutions as test reactions. Representative data for octan-2-ol, *rac*-**1d** and 1-phenylethanol, *rac*-**1a** with each biocatalysts are shown. [Lipase AK (TEOS:OcTEOS:DMDEOS 4:1:1, 2×, no additive); Lipase PS (TEOS:OcTEOS:DMDEOS 4:1:1, 2×, 0.5 v/v% LA); CaLB (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, 0.5 v/v% BRIJ); CrL (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, 0.5 v/v% BRIJ)]. The reaction times were 1 h for Lipase AK, Lipase PS and CaLB and 4 h for CrL in the batch mode in hexane:MTBE 2: solvent (for further reaction details, see Materials and Methods section and Appendix, Annex 16).

To demonstrate the synthetic capabilities of our imprinted sol-gel lipases in real kinetic resolution, the enzymatic acylation of the less examined racemic 1-(thiophen-2-yl)ethanol *rac*-**1h** was chosen (Figure 26). First, the kinetic resolution of this alcohol *rac*-**1h** was investigated with all the four ternary systems [TEOS:OcTEOS:PhTEOS 10:7:3 (1× and 2× enzyme loading) and TEOS:OcTEOS:DMDEOS 4:1:1 (1× and 2× enzyme loading)] for the four imprinted lipases (Lipase AK without additive, Lipase PS with LA, CrL and CaLB with BRIJ) (Figure 28 and Table 18). Comparison of the the productivity (specific reaction rate, *r*) data revealed again that the biocatalysts were always more productive in continuous-flow system (gray bars in Figure 28) than in the corresponding batch mode reactions (black bars in Figure 28).

Results with the lipases of sufficient enantiomer selectivity (*E*) in kinetic resolution of racemic 1-(thiophen-2-yl)ethanol *rac*-**1h** indicated that higher enzyme loading resulted in more active immobilized biocatalysts (Table 18). Not surprisingly, the same ternary sol-gel biocatalysts (TEOS:PhTEOS:DMDEOS 4:1:1, high lipase loading) proved to be the most efficient in the single racemate (*rac*-**1h**) based kinetic resolutions which were the best in the multiple substrate tests (Table 17).

Table 18. Comparison of lipases imprinted with the best performing additives as immobilized biocatalysts in ternary sol-gel matrices in batch mode kinetic resolutions of 1-(thiophen-2-yl)ethanol *rac*-**1h**. The specific activity (U_B) and enantiomer selectivity (E) of the free and immobilized forms of lipases are shown. (Reaction time 4 h, in hexane:MTBE; 2:1, for further reaction details, see Materials and Methods section).

Silane precursors (enzyme amount)	**Lipase AK**[a]		**Lipase PS**[a]		**CaLB**[a]	
	U_B (U g^{-1})	E[b]	U_B (U g^{-1})	E[b]	U_B (U g^{-1})	E[b]
-[c]	70	>100	28	>100	70	»200
TEOS:OcTEOS:PhTEOS 10:7:3 (1×)[d]	23	90	44	>100	25	>100
TEOS:OcTEOS:PhTEOS 10:7:3 (2×)[e]	71	89	73	73	66	>200
TEOS:PhTEOS:DMDEOS 4:1:1 (1×)[d]	31	81	33	>100	23	>100
TEOS:PhTEOS:DMDEOS 4:1:1 (2×)[e]	70	»200	70	>100	54	>200

[a] Imprinting additives: no additive for Lipase AK, LA (0.5 v/v%) for Lipase PS, BRIJ for (0.5 v/v%) for CaLB.

[b] The enantiomer selectivity (E) was calculated from c and $ee_{(R)\text{-}\mathbf{2}}$ (CHEN et al. 1982). Due to sensitivity to experimental errors, E values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

[c] The corresponding commercially available native lipase powder for Lipase AK and Lipase PS. For CaLB, the commercial liquid enzyme (Novozym CALB L) was immobilized on Novozym carrier (1 mg carrier to 8 µl enzyme solution).

[d] Normal (single) enzyme loading (for further details, see Methods section).

[e] Double enzyme loading (for further details, see Methods section).

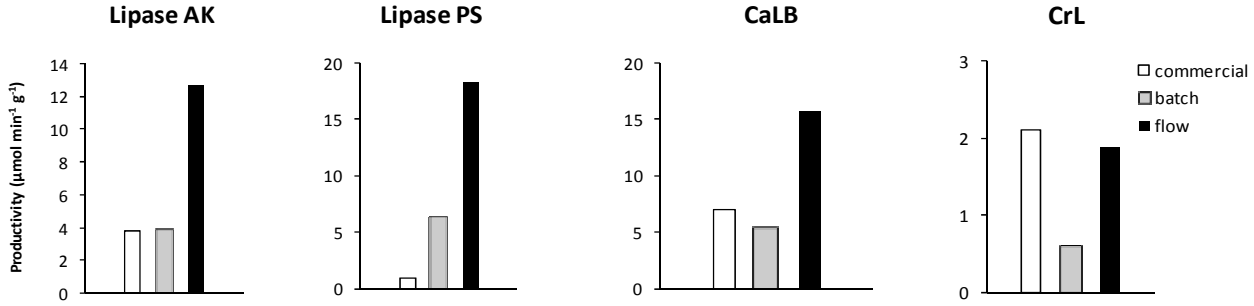


Figure 28. Comparison of the productivity (specific reaction rate, r) for the best performing imprinted sol-gel lipases in batch mode and in continuous-flow packed-bed reactor in kinetic resolution of 1-(thiophen-2-yl)ethanol *rac*-**1h**. [Lipase AK (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, no additive); Lipase PS (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, 0.5 v/v% LA); CaLB (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, 0.5 v/v% BRIJ); CrL (TEOS:PhTEOS:DMDEOS 4:1:1, 2×, 0.5 v/v% BRIJ)].

Finally, the two most selective sol-gel entrapped lipases (Lipase AK, 2× loading in TEOS:PhTEOS:DMDEOS 4:1:1 system without additive and CaLB, 2× loading in TEOS:PhTEOS:DMDEOS 4:1:1 system with BRIJ) were applied to perform the kinetic resolutions of 1-(thiophen-2-yl)ethanol *rac*-**1h** in continuous-flow reactors on preparative scale (Table 19). Although the enantiomer selectivity for both biocatalysts was reasonably high in the batch mode tests (E >200, see Table 18), the enantiomeric excess of the isolated acetate (*R*)-**2h** from the preparative scale continuous-flow experiment was rather low. In a next experiment, however, the (*S*)-**1h** of 99.2 %ee was acetylated quantitatively to (*S*)-**2h** using the non-selective CaLA in continuous-flow reaction. The fact, that (*S*)-**2h** had only 92.0 %ee after the same chromatographic purification indicated the low enantiomeric excesses of the acetates (*R*)- and (*S*)-**2h** are rather due to the sensitivity of the product to racemization than the insufficient selectivity of the lipases in the continuous-flow system.

Table 19. (*S*)-1-(thiophen-2-yl)ethanol (*S*)-**1h** and (*R*)-1-(thiophen-2-yl)ethyl acetate (*R*)-**2h** prepared by kinetic resolutions with sol-gel immobilized imprinted lipases in continuous-flow reactors. [*rac*-**1h**, 5 mg mL^{-1} in hexane:TBME:vinyl acetate 6:3:1, 0.2 mL/min, 24 h flow time]

Compound	**Lipase** (precursors, additive, enzyme amount; temperature)	Y[a] (%)	*ee*[b] (%)
(*S*)-**1h**	Lipase AK (TEOS:PhTEOS:DMDEOS 4:1:1, -, 2×; 30 °C)	35.0	99.2
(*R*)-**2h**	CaLB (TEOS:PhTEOS:DMDEOS 4:1:1, BRIJ, 2×; 30 °C)	20.8	88.8
(*S*)-**2h**[c]	CaLA (immobilized on IB-150 carrier; 50 °C)[c]	95.8	92.0

[a] Yields refer to isolated products after chromatographic separation.
[b] The *ee* values were determined by enantioselective GC.
[c] Non-selective acylation with full conversion from (*S*)-**1h** of 99.2 %ee using immobilized CaLA-filled bioreactor.

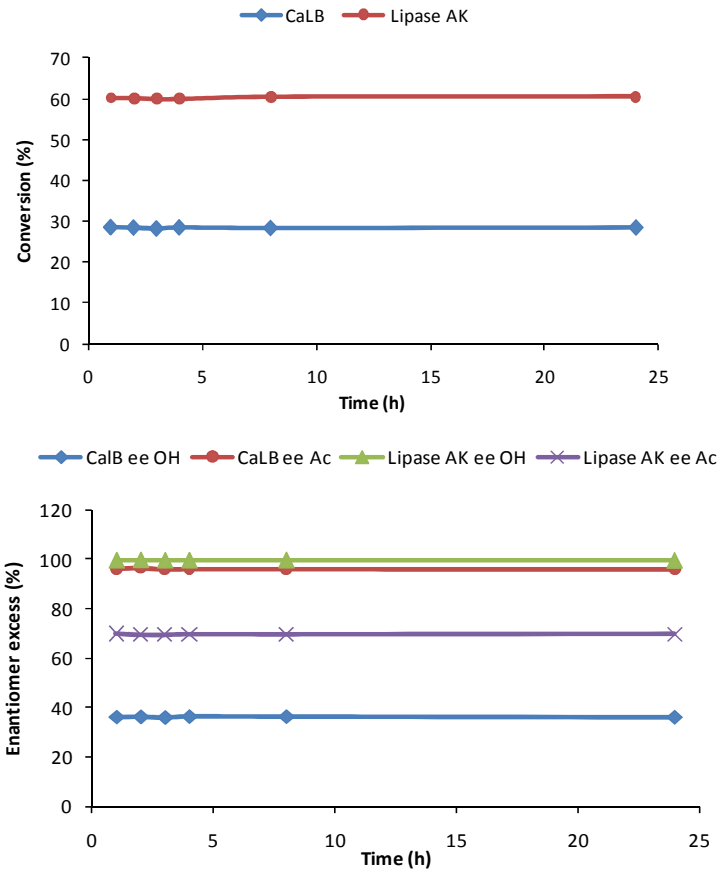


Figure 29. Demonstration of sol-gel bioimprinted CalB (BRIJ) and Lipase AK (-) stability in continuous-flow for 24 h on conversion (%) and enantiomer excess (%) of preparative kinetic resolutions reactions

It was demonstrated during continuous-flow operations that our biocatalysts, CaLB imprinted with BRIJ 30 and Lipase AK without additive were impressively stabile in a 24h time period (Figure 29).

Based on the results of experiments the followings can be concluded. As a new application of biocatalysts one-pot synthesis is attractive for many reasons including the minimization of handling procedures, consequent reduction in reagent use, and gains in volumetric productivity.

As it already has been demonstrated, *trans*-free fat alternatives for various food applications by enzymatic routes are possible (CAMP et al. 1998, YANKAH and AKOH 2000). Thus, it can be assumed that a fully enzymatic process that avoids chemical interesterification may be superior to the existing methods (MOREAU et al. 2002). Consequently, the physical and physiological properties of enzyme-made "value-added" products are expected to be identical or superior to the blend of chemically made structured lipids and phytosterol esters.

Screening lipases and sterol esterases as biocatalysts for enzymatic one-pot processes to produce mixtures of specific structured lipids and phytosterol esters starting from a mixture of phytosterol, caprylic acid and sunflower oil revealed that this valuable food component could be prepared purely enzymatically in organic-solvent-free medium. The process applied an esterification reaction of phytosterol with free fatty acids catalyzed by sterol esterase of *Aspergillus oryzae* and lipase catalyzed transesterification with immobilized lipase from *Mucor miehei* (Lipozyme) resulted in 92.1% conversion to phytosterol ester and 44.1% conversion to MLM / MML triacylglycerols. The final product could be obtained by simple filtration of the biocatalysts from the reaction product followed by removal of the free fatty acids by vacuum distillation. Because chemical transesterification has been avoided, this process resulted in no increase in the initial *trans*-fatty acid content of the mixture.

As the integrated enzymatic process developed for the production of structured lipid - phytosterol ester blends applies only food-grade biocatalysts, suitable industrial applications of the fully enzymatically produced compositions in foods, beverages, pharmaceuticals and nutraceuticals could provide an alternative way to combat obesity by improving the quality of lipid intake (ST-ONGE et al. 2003, AKOH and LAI 2005).

The beneficial properties of the sol-gel immobilized lipases may be significantly influenced by the nature of the R' substituent in binary sol-gel systems of trialkoxy- and tetraalkoxysilane precursors [R'-$Si(OR)_3$ and $Si(OR)_4$] (REETZ et al. 1995, KAWAKAMI and YOSHIDA 1996) but ternary sol-gel systems of a tetraalkoxysilane and two different trialkoxysilane precursors [R^1-$Si(OR)_3$, R^2-$Si(OR)_3$ and $Si(OR)_4$] had not been studied for lipase immobilization.

The lipase from *Pseudomonas fluorescens* (Lipase AK) proved to be a good lipase model for studies of factors influencing the sol-gel immobilization of lipases. (PÉTER et al. 2005, ZARCULA et al. 2009). Because the esterification activity of immobilized lipases can be enhanced by forming the entrapping-gel on the surface of Celite (KAWAKAMI and FURUKAWA 1996, FURUKAWA

and KAWAKAMI 1998), fine tuning the sol-gel immobilization process of Lipase AK deposited on Celite 545 was selected as the subject for this study. Thus, the properties of Celite-supported Lipase AK immobilized with ternary silane precursor systems of various alkyltriethoxysilanes (alkylTEOS), phenyltriethoxysilane (PhTEOS) and tetraethoxysilane (TEOS) were investigated in this work.

Effects of the surface coverage and fine-tuning properties were investigated for the sol-gel entrapment of Celite-supported Lipase AK.

SEM investigations showed that preparations of lower Lipase AK:Celite ratio (1:10 and 1:4) had uniform and thin enzyme coverage, whereas higher enzyme loading (1:2, 3:4 or 1:1 ratios) resulted in thick enzyme layers containing large crystal-like particles. The enzyme-containing layer remained uniform and thin after the sol-gel entrapment of the Celite-supported Lipase AK of low enzyme loading (1:10) resulting in significant increases in activity yields (Y_A > 200 %) by lowering the diffusion limitations which are more pronounced in the larger aggregates of free Lipase AK.

The study of the catalytic behavior of binary and ternary sol-gel biocatalysts in kinetic resolutions of 1-phenylethanol *rac*-**1a** and 2-heptanol *rac*-**1b** using silane precursor systems consisting of alkylTEOS, PhTEOS and TEOS indicated the possibility of fine-tuning with and the importance of ternary sol-gel systems for lipase entrapment. For both substrates *rac*-**1a**,**b**, there were found one or more alkylTEOS : PhTEOS : TEOS ternary systems which surpassed the catalytic properties of any alkylTEOS : TEOS or PhTEOS : TEOS binary systems. Our study also indicated that different sol-gel compositions were optimal for the two different substrates. Although the medium-chain octylTEOS- and perfluorooctylTEOS-containing ternary systems performed the best in general, this study indicated that individual fine-tuning might develop the best biocatalyst for each individual substrate.

The study on bioimprinting effects of substrate-mimicking molecules selected systematically with the aid of experimentally verified cases in sol-gel immobilization of lipases with binary and ternary silane precursor compositions indicated that independently from the nature of sol-gel matrix, the most pronounced imprinting effects were found with such additives which were found mimicking the substrates in the experimental structures of the lipases. Our case study revealed, that lauric acid being the most effective imprinting additive for lipase PS (the crystal structure of lipase from *Burkholderia cepacia* included stearic acid) while in case of *Candida* species (CaLB, CrL) tetraethylene glycol dodecyl ether (BRIJ 30) exhibited the most significant imprinting effect (the porcine pancreatic lipase resembling similarity to CrL included tetraethylene glycol octyl ether and the crystal structure of CaLB included methylpenta(oxyethyl)heptadecanoate).

The high efficiency of sol-gel systems containing trialkoxysilanes OcTEOS or PhTEOS may be explained by assuming these silanes or their partially hydrolyzed forms having imprinting effect as well. This can rationalize also why the most efficient biocatalysts from Lipase AK (lipase from *Psudomonas fluorescens*) did not contain imprinting additives.

The rational selection of imprinting molecules can be combined with proper compositions of silane precursors in large scale production of sol-gel immobilized lipases for various applications. The robust sol-gel entrapped forms of the selected four lipases (from Lipase AK, Lipase PS, lipase B from *Candida antarctica* and *Candida rugosa* lipase) proved to be ideal biocatalysts in biotransformations such as kinetic resolution of a racemic alcohols in batch and continuous-flow systems.

6. NEW SCIENTIFIC RESULTS

I. Enzymatic one-pot synthesis of specific structured lipids and phytosterol esters.

T1. It was revealed that specific structured lipid and phytosterol ester mixture as valuable food component could be prepared starting from natural compounds in an enzyme catalyzed one-pot synthesis under solvent-free conditions.

T2. It was stated/concluded that the best process variant using a sequence of sterol esterase (*Ao*SSF)-catalyzed esterification reaction of the free fatty acids and phytosterols, followed by water removal in vacuum and lipase-catalyzed transesterification with immobilized lipase from *Rhizomucor miehei* (Lipozyme) resulted in 92.1% conversion to phytosterol esters and 44.1% conversion to triacylglycerols containing two caprylic esters (MLM/MML). Furthermore as chemical transesterification had been avoided, this process resulted in no increase in the initial trans-fatty acid content of the mixture.

II. Fine-tuning the precursor composition for sol-gel immobilization of lipases

T3. It was revealed by SEM investigations that preparations of lower Lipase AK:Celite ratio (1:10 and 1:4) had uniform and thin enzyme coverage, whereas higher enzyme loading (1:2, 3:4 or 1:1 ratios) resulted in thick enzyme layers containing large crystal-like particles. The enzyme-containing layer remained uniform and thin after the sol–gel entrapment of the Celite-supported Lipase AK of low (1:10) resulting in significant increases in activity yields (Y_A>200%) by lowering the diffusion limitations which are more pronounced in the larger aggregates of free Lipase AK.

T4. The importance of fine tuning was confirmed by the ternary sol-gel systems as the catalytic properties of the best ternary compositions were superior to any of the binary systems.

It was indicated that among binary systems the TEOS:PhTEOS=1:1 composition resulted in optimal properties regarding both activity and selectivity. Thus these results were used in the fine-tuning of the ternary systems.

It was revealed the best ternary composition can be prepared from HexTEOS, OctTEOS, PFOctTEOS precursors, while enantiomer selectivities were sufficient for almost all the longer alkylTEOS precursors (HexTEOS, OctTEOS, PFOctTEOS, DecTEOS, DodTEOS, OctdTEOS). Among all the ternary systems, the perfluorinated chain containing PFOctTEOS series exhibited the best overall

performance. Taking the price of PFOctTEOS also into account, however, the TEOS: OcTEOS:PhTEOS system provided the best performance / price result in the kinetic resolution reactions of 1-phenylethanol.

T5. Successful improvements in the supported sol-gel immobilization of Lipase AK were developed. The new biocatalysts prepared by our immobilization methods showed better catalytic properties then the commercial sol-gel lipases. The activity yields (Y_A) of our biocatalysts showed upto 1.4-fold activity yield increase (Y_A were varied between 109-242 %) compared to the commercial ones.

III. Preparation of novel sol-gel lipases by designed bioimprinting

T6. It was verified that rationally selected substrate mimicking molecules resembling to those found in experimental X-ray structures of lipases showed the most pronounced imprinting effects. Lauric acid was the most effective imprinting additive for Lipase PS (the crystal structure of lipase from *Burkholderia cepacia* included stearic acid) while in case of *Candida* species (CaLB, CrL) tetraethylene glycol dodecyl ether (BRIJ 30) exhibited the most significant imprinting effect (the porcine pancreatic lipase resembling similarity to CrL included tetraethylene glycol octyl ether and the crystal structure of CaLB included methylpenta(oxyethyl)heptadecanoate).

T7. It was concluded that the high efficiency of sol-gel systems containing trialkoxysilanes OcTEOS or PhTEOS may be explained by assuming these silanes or their partially hydrolyzed forms have an imprinting effect as well. This assumption can explain why Lipase AK (lipase from *Psudomonas fluorescens*) required no imprinting additives for maximal performance.

T8. A novel method for testing the biocatalytic properties of the immobilized lipases was developed based on enentioselective GC analysis of the lipase-catalyzed acylation reactions of racemic multisubstrate mixtures in batch and in continuous-flow reactors. It was the first application of multisubstrate systems for comparison of productivity and selectivity of sol-gel entrapped enzymes.

T9. The biocatalytic usefulness of the two most selective sol-gel entrapped lipases (Lipase AK, 2× loading in TEOS:PhTEOS:DMDEOS 4:1:1 system without additive and CaLB, 2× loading in TEOS:PhTEOS:DMDEOS 4:1:1 system with BRIJ) was confirmed by the kinetic resolution of racemic 1-(thiophen-2-yl)ethanol (*rac*-**1h**) in batch and continuous-flow systems reactors on preparative scale.

7. SUMMARY

As it was targeted in the objectives, my thesis work focused mainly on biocatalysis which is an effective tool for providing environmentally friendly processes for all life science industries (pharma, food, feed, agro). In the first part of the thesis, the integrated enzymatic production of specific structured lipids and phytosterol esters composition has been discussed. In which a special emphasis was given to the usage of starting materials from natural sources and using solely enzymatic methods in organic solvent-free media to avoid the increase of the unwanted *trans*-fatty acid content. This was rather the application side of biocatalysis.

Hereafter my attention was shifted toward a more challenging part that is how to engineer biocatalysts from enzymes as biological entities to industrial reactors. As it has been known that preparing effective, suitable biocatalysts is very important as the catalytic activity, selectivity, specificity and enzyme stability are the key factors affecting biocatalysis efficiency. Thus the second major part was branching in two from this common root. In our first experiments within this topic attempted to carry out a systematic study on sol-gel immobilization. Since earlier results showed that it is an easy and effective way to create robust biocatalysts with improved stability and activity thus this was selected for further optimization. The systematic studies were carried out on *Pseudomonas fluorescens* lipase (Lipase AK). The method (normal and composite sol-gel respectively) was extended for Lipase PS and different carriers as well using ternary silane precursor systems of various alkyltriethoxysilanes. Improvements of the sol-gel immobilization method of lipases were successfully developed. The new biocatalysts prepared by our immobilization methods showed better catalytic properties then the commercial lipases prepared by similar methods.

Finally an interesting and unconventional immobilization method, bioimprinting effect in sol-gel immobilization of lipases was studied to develop efficient novel immobilized biocatalysts with significantly improved properties for biotransformations in continuous-flow systems. Four lipases were immobilized by sol-gel process with nine bioimprinting candidates using various combinations of silica precursors. The biocatalytic properties of the immobilized lipases were characterized by enantiomer selective acylation of various racemic secondary alcohols in multisubstrate systems. The most significant activity enhancement was found with those imprinting molecules which were found mimicking the substrates in the experimental structures of the lipases. The biocatalytic usefulness of the best preparations was demonstrated by the kinetic resolution of racemic 1-(thiophen-2-yl)ethanol in batch and continuous-flow systems.

As a future perspective it can be suggested that the rationally selected imprinting molecules can be combined with proper supporting materials such as silica-gel in large scale production of sol-gel immobilized lipases for various applications.

7. ÖSSZEFOGLALÁS

Doktori értekezésemben a biokatalízis vagyis enzimkatalizált módszerekre helyeztem a hangsúlyt, melyek hatékony eszközként lehetővé teszik a környezetbarát technológia alkalmazását a gyógyszeripar, élelmiszeripar, és mezőgazdaság területén egyaránt. Dolgozatom első fő fejezetében specifikus strukturált lipid és növényi szterinészter keverék integrált enzimes előállítását tűztem ki célul. Különös hangsúlyt fektettem a kiindulási anyagok természetes eredetére és a nemkívánatos transz−zsírsavak képződésének visszaszorítására, melyet az alkalmazott kizárólag enzimatikus módszerrel, szerves oldószermentes közegben sikerült megvalósítanom.

Értekezésem második részében nagyobb kihívást magában rejtő témakör felé fordultam, és azt vizsgáltam, hogyan fejlesszünk enzimekből ipari reaktorokban felhasználható biokatalizátorokat. Hatékony, alkalmazás-specifikus biokatalizátor fejlesztése nagy jelentőséggel bír, mivel a katalitikus aktivitás, szelektivitás, specifitás és enzimstabilitás mind kulcsszerepet játszanak a biokatalízis hatékonyságában.

Ezen témakörön belül először szisztematikus (prekurzor / hordozó / rögzítési körülmények / adalékok hatásai) szol-gél enzimrögzítési kísérleteket végeztünk, mivel korábbi eredményeink azt mutatták, hogy ez egy könnyen kivitelezhető hatékony, robusztus biokatalizátorok előállítására alkalmas módszer. A lipáz enzimek szol-gél rendszerbe történő stabilizálásával a biokatalizátor eltarthatósága, hőstabilitása és újrafelhasználhatósága megnő, így a további optimalizálási kísérleteink megvalósításához ezt a módszert választottuk.

A szisztematikus vizsgálatok összehasonlíthatósága miatt a kísérleteket zömében a *Pseudomonas fluorescens* által termelt lipázzal (lipáz AK) végeztük. A módszer (normál, ill. kompozit szol-gél) más lipázra (lipáz PS, CrL) és hordozóra történő kiterjesztésével is foglalkoztunk. Eredményesen fejlesztettük tovább egyes lipázok szol-gél rendszerben történő immobilizálását. Az általunk elkészített, új, rögzített enzimkészítmények katalitikus tulajdonságai meghaladják a kereskedelmi forgalomban kapható hasonló módon rögzített biokatalizátorokét.

Végül egy érdekes, nem megszokott enzimrögzítési módszer, a bioimprinting szol-gél rögzítésre gyakorolt hatásait vizsgáltuk. Négyféle lipázt rögzítettünk kilencféle bioimprinting molekulával a szilánkomponensek változtatása mellett. A rögzített lipázok biokatalitikus tulajdonságát enantiomer szelektív, racém szekunder alkoholok multiszubsztrát rendszerben történő kinetikus rezolválási reakciójával teszteltük. A legjelentősebb aktivitásnövekedést azon imprinting molekulák esetében tapasztaltuk, amelyek eredetileg is megtalálhatóak voltak a lipázok kristályszerkezetében. A legjobb, bioimprintinggel előállított biokatalizátorok hatékonyságát egy korlátozottan szelektív racém alkohol, a tiofén, szakaszos és folyamamtos üzemű acilezési reakcióján keresztül vizsgáltuk.

Javaslatként, távlati tervként eredményeink továbbfejlesztése céljából a racionálisan kiválasztott imprinting molekulák szol-gél rendszerben való rögzítését lehetne ötvözni a hordozóhoz, például szilikagélhez történő rögzítéssel, amely elméletünk szerint még robusztusabb, stabilabb és hőstabilabb biokatalizátort eredményezne.

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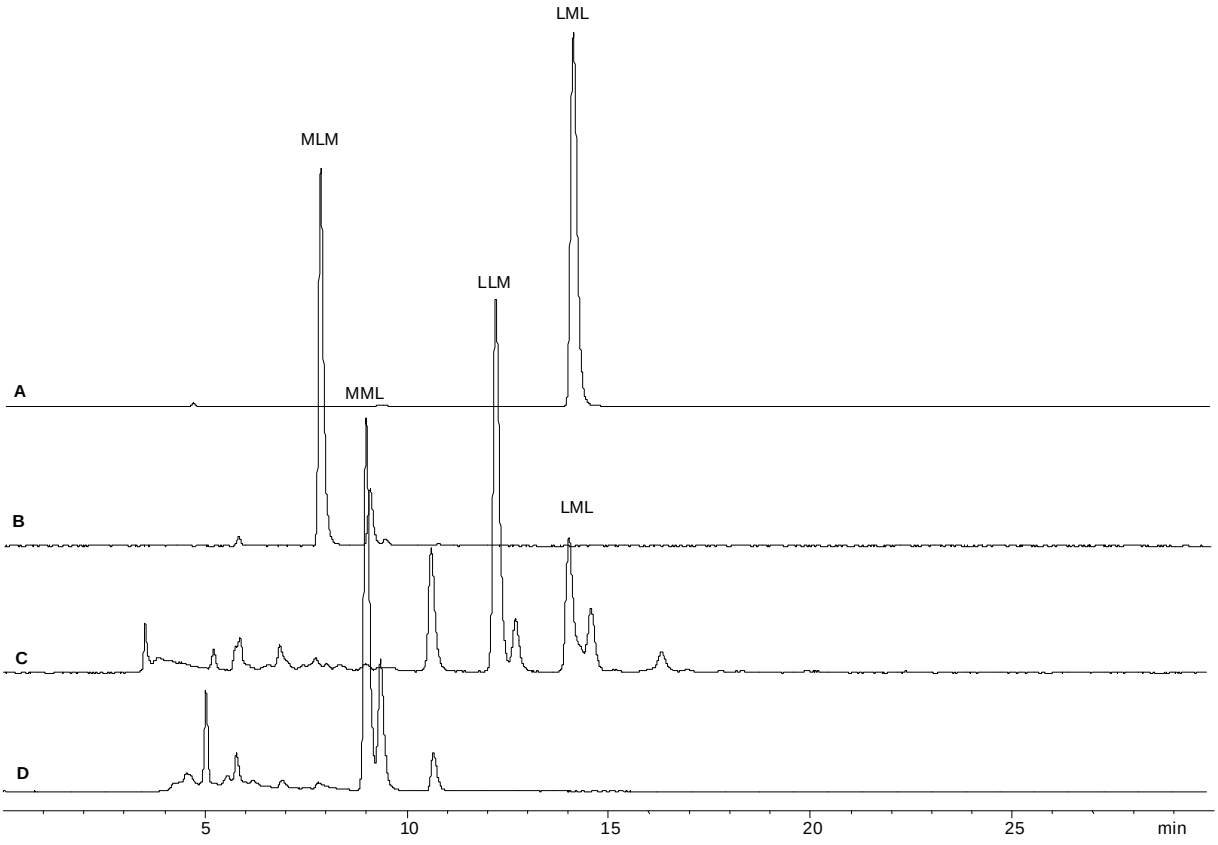
9. APPENDIX

Annex 1. HPLC chromatograms of the synthetic structured triglyceride standards. A: LML; B: MLM; C: LLM; D: MML



Annex 2. NMR spectral data

Dodecan-2-yl-acetate

^{1}H NMR (300 MHz, $CDCl_3$-d6): δ 0.88 (*J* 5.84; 3H, t, CH_3), 1.20 (*J* 6.21; 3H, d, CH_3), 1.27 (14H, m, 7×CH_2), 1.43-1.61 (*2*H, m, CH_2), 2.03 (3H, s, CH_3), 4.82-4.95 (1H, m, CH); ^{13}C NMR (75 MHz, $CDCl_3$-d6): δ 14.28 (CH_3), 20.15 (CH_3), 21.57 (CH_3), 22.85 (CH_2), 25.60 (CH_2), 29.43 (2× CH_2), 29.66 (2×CH_2), 29.80 (CH_2) 33.06 (CH_2), 36.14 (CH_2), 71.28 (CH), 170.97 (C);

Decan-2-yl-acetate

^{1}H NMR (300 MHz, $CDCl_3$-d6): δ 0.89 (*J* 6.40; 3H, t, CH_3), 1.20 (*J* 7.16; 3H, d, CH_3), 1.27 (12H, m, 6×CH_2), 1.41-1.61 (2H, m, CH_2), 2.03 (3H, s, CH_3), 4.88-4.95 (1H, m, CH); ^{13}C NMR (75 MHz, $CDCl_3$-d6): δ 14.11 (CH_3), 19.97 (CH_3), 21.39 (CH_3), 22.70 (CH_2), 25.43 (CH_2), 29.34 (3×CH_2), 31.93 (CH_2), 35.97 (CH_2), 71.10 (CH), 170.80 (C);

Nonan-2-yl-acetate

^{1}H NMR (300 MHz, $CDCl_3$-d6): δ 0.88 (*J* 6.03; 3H, t, CH_3), 1.20 (*J* 6.22; 3H, d, CH_3), 1.27 (10H, m, 5×CH_2), 1.41-1.64 (2H, m, CH_2), 2.03 (3H, s, CH_3), 4.83-4.96 (1H, m, CH); ^{13}C NMR (75 MHz, $CDCl_3$-d6): δ 14.27 (CH_3), 20.16 (CH_3), 21.58 (CH_3), 22.83 (CH_2), 25.61 (CH_2), 29.40 (CH_2), 29.62 (CH_2), 31.99 (CH_2), 36.14 (CH_2), 71.29 (CH), 171.00 (C);

Octanol-2-yl-acetate

^{1}H NMR (300 MHz, $CDCl_3$-d6): δ 0.89 (*J* 5.65; 3H, t, CH_3), 1.21 (*J* 6.22; 3H, d, CH_3), 1.28 (8H, m, 4×CH_2), 1.44-1.62 (2H, m, CH_2), 2.03 (3H, s, CH_3), 4.84-4.95 (1H, m, CH); ^{13}C NMR (75 MHz, $CDCl_3$-d6): δ 14.26 (CH_3), 20.15 (CH_3), 21.59 (CH_3), 22.81 (CH_2), 25.57 (CH_2), 29.33 (CH_2), 31.95 (CH_2), 36.14 (CH_2), 71.28 (CH), 171.00 (C);

Heptan-2-yl-acetate

^{1}H NMR (300 MHz, $CDCl_3$-d6): δ 0.89 (*J* 5.65; 3H, t, CH_3), 1.20 (*J* 5.65; 3H, d, CH_3), 1.28 (6H, m, 3×CH_2), 1.40-1.61 (2H, m, CH_2), 2.02 (3H, s, CH_3), 4.84-4.95 (1H, m, CH); ^{13}C NMR (75 MHz, $CDCl_3$-d6): δ 14.16 (CH_3), 20.14 (CH_3), 21.56 (CH_3), 22.73 (CH_2), 25.25 (CH_2), 31.83 (CH_2), 36.07 (CH_2), 71.25 (CH), 170.97 (C);

Hexan-2-yl-acetate

^{1}H NMR (300 MHz, $CDCl_3$-d6): δ 0.89 (*J* 6.78; 3H, t, CH_3), 1.20 (*J* 6.21; 3H, d, CH_3), 1.29 (4H, m, 2×CH_2), 1.43-1.62 (2H, m, CH_2), 2.02 (3H, s, CH_3), 4.84-4.95 (1H, m, CH); ^{13}C NMR (75 MHz, $CDCl_3$-d6): δ 13.98 (CH_3), 19.97 (CH_3), 21.39 (CH_3), 22.55 (CH_2), 27.59 (CH_2), 35.66 (CH_2), 71.08 (CH), 170.81 (C);

1-(Thiophen-2-yl)ethanol

^{1}H NMR: 1.62 (*J1* 4.71; *J2* 1.70; 3H, dd, CH3), 2.11 (1H, s, OH), 5.15 (*J* 6.22; 1H, q, CH), 6.96-7.28 (3H, m, Ar-CH);

^{13}C NMR: 25.45 (CH3), 66.45 (CH), 123.38 (CH), 124.62 (CH), 126.86 (CH), 150.08 (C);

1-(Thiophen-2-yl)ethyl acetate

^{1}H NMR: 1.66 (*J* 6.59; 3H, d, CH3), 2.08 (3H, s, CH3), 6.19 (*J* 6.41; 1H, q, CH), 6.97-7.29 (3H, m, Ar-CH);

^{13}C NMR: 21.49 (CH3), 22.24 (CH3), 67.75 (CH), 125.39 (CH), 125.46 (CH), 126.77 (CH), 144.71 (C), 170.42 (C=O).

1-Phenylethyl acetate

^{1}H NMR: 1.57 (*J* 6.59; 3H, d, CH3), 2.10 (3H, s, CH3), 5.92 (*J* 6.59; 1H, q, CH), 7.28-7.39 (5H, m, Ar-CH)

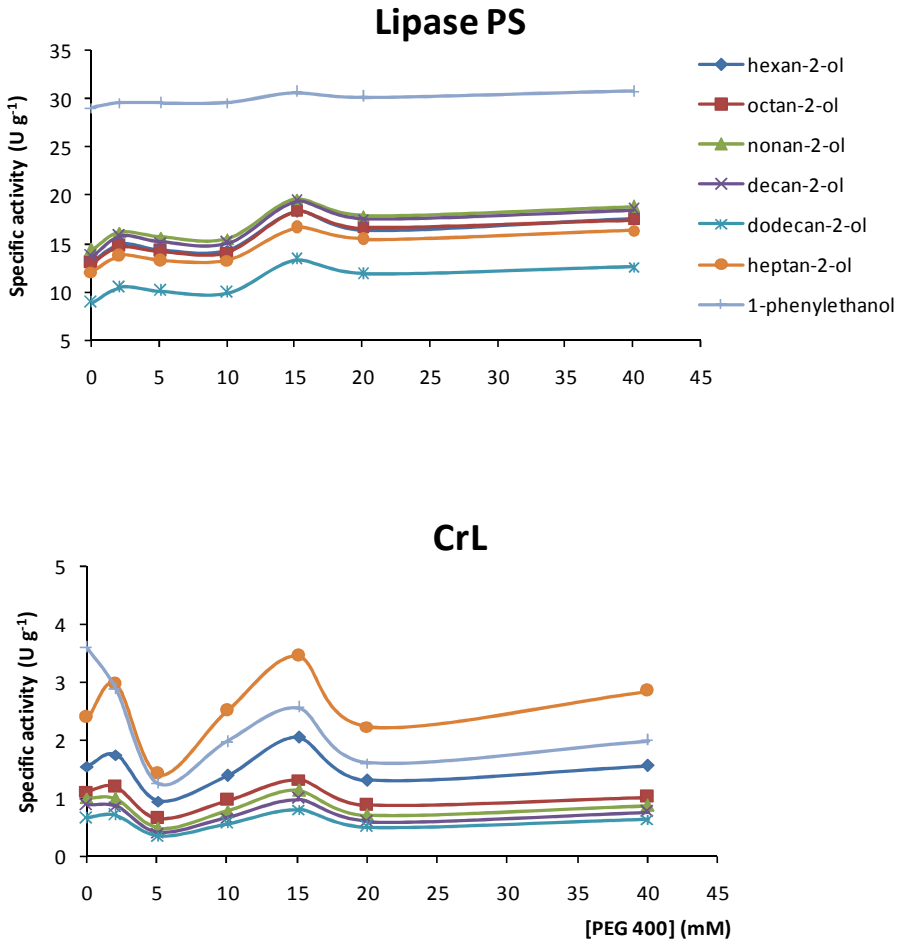
^{13}C NMR: 21.38 (CH3), 22.25 (CH3), 72.35 (CH), 126.14 (2×CH), 127.91 (CH), 128.54 (2×CH), 141.75 (C), 170.33 (C=O)

Tetraethylene glycol dilauroyl ester,(L-TEG-L)

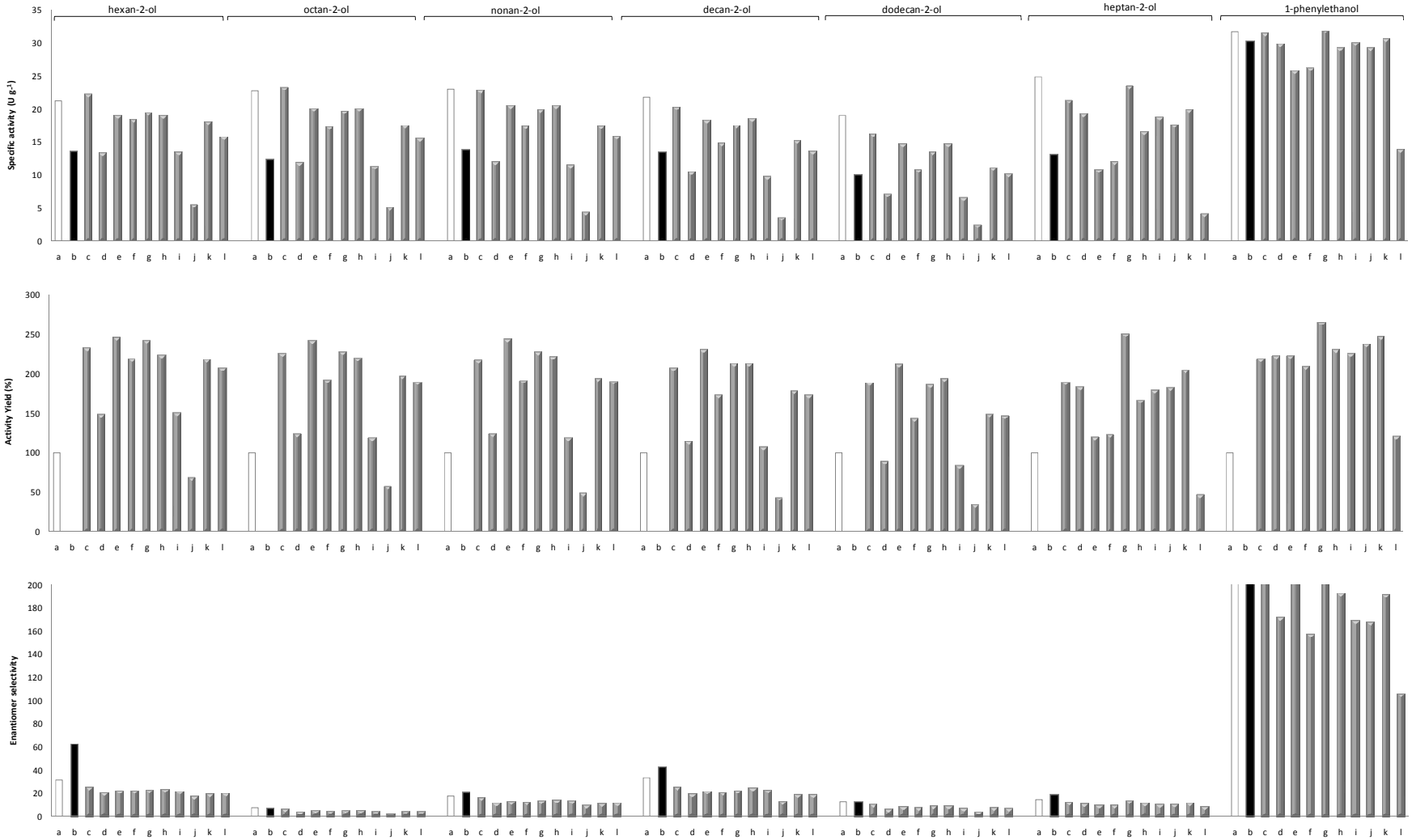
^{1}H NMR: 0.89 (*J* 6.97; 6H, t, 2×CH3), 1.27 (32H, s, 16×CH2), 1.63 (*J* 6.97; 4H, m, 2×CH2), 2.34 (*J* 7.54; 4H, t, 2×CH2), 3.67 (8H, s, 4×CH2), 3.71 (*J* 4.90; 4H, t, 2×CH2), 4.24 (*J* 4.71, 4H, t, 2×CH2);

^{13}C NMR: 14.31 (2×CH3), 22.88 (2×CH2), 25.11 (2×CH2), 29.35 (2×CH2), 29.45 (2×CH2), 29.53 (2×CH2), 29.67 (2×CH2), 29.81 (4×CH2), 32.11 (2×CH2), 34.42 (2×CH2), 63.54 (2×CH2), 69.43 (2×CH2), 70.77 (2×CH2), 70.84 (2×CH2), 174.06 (2×C=O).

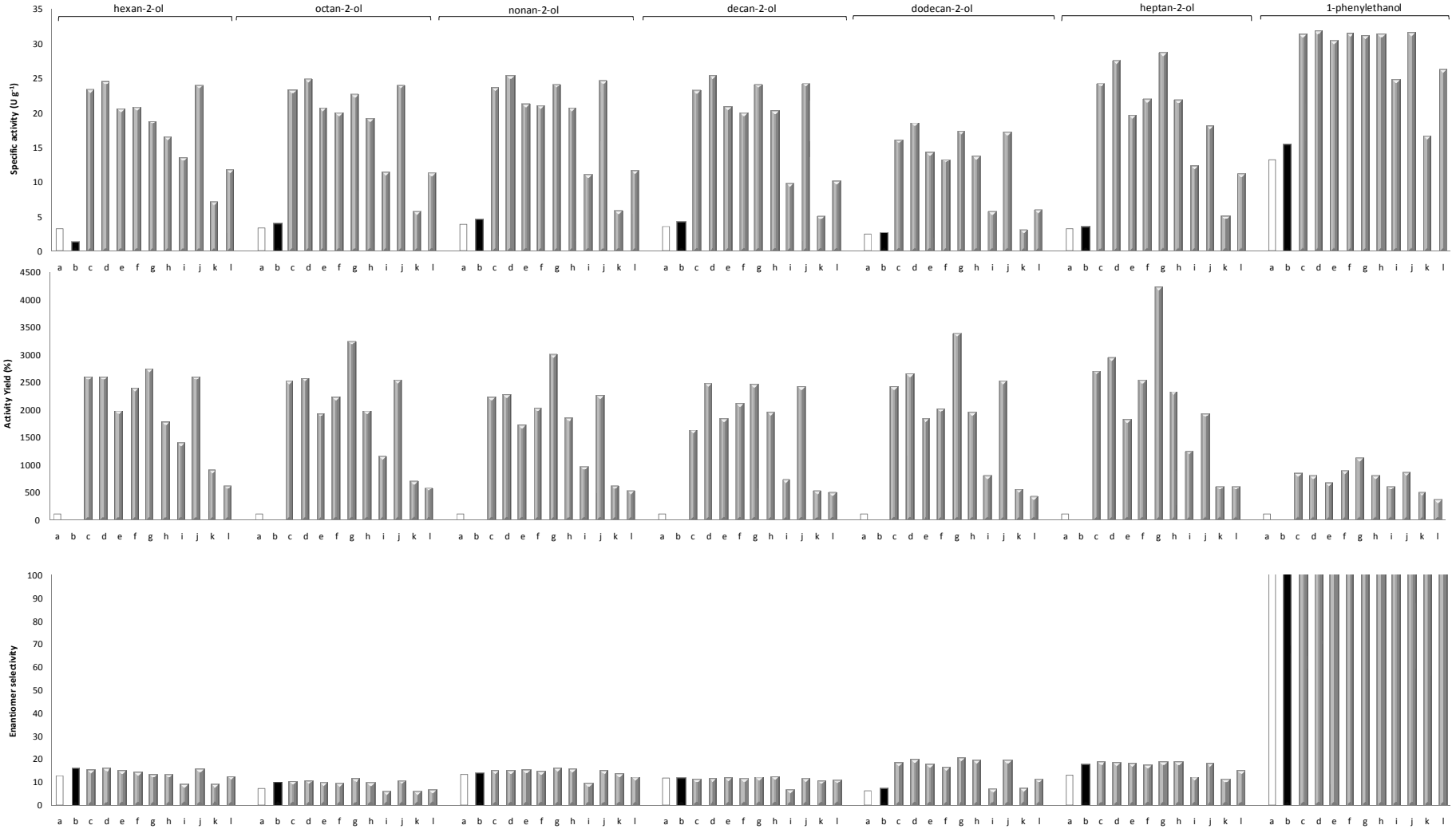
Annex 3. Dependence of the specific activity (U_B, U g^{-1}) on the concentration of PEG 400 as imprinting additive for Lipase PS (2h) and CrL (8h) (referring to Figure 24 in Results and Discussion)



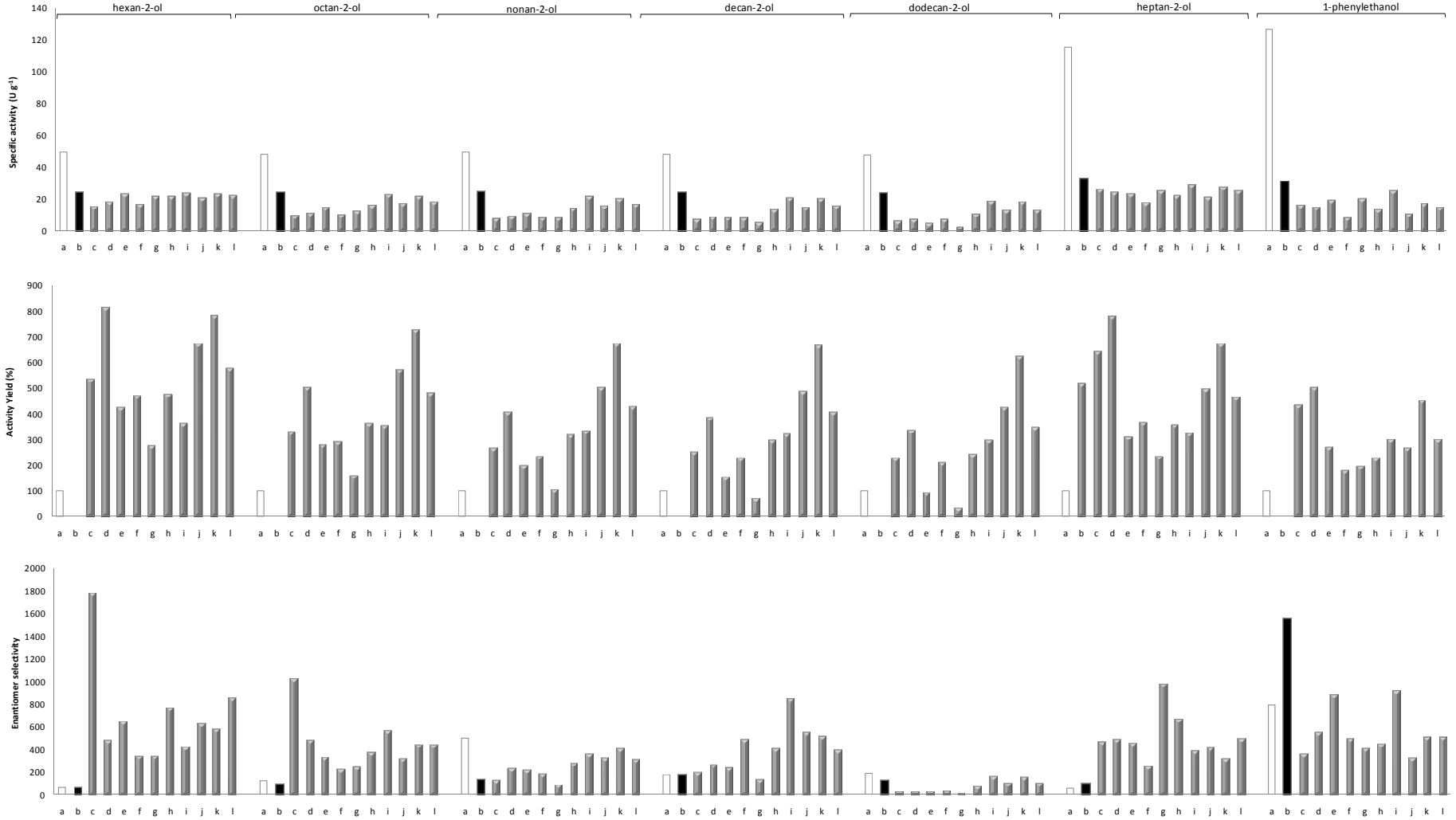
Annex 4. Screening of the additives for bioimprinting in batch mode and characterization of sol-gel immobilized bioimprinted lipase AK in binary sol-gel systems of TEOS: PhTEOS silane precursors. Specific activity (U_B) activity yield (Y_A) and enantiomer selectivity (E) of the free and immobilized forms Data of LAK represent 2hrs.
[a] corresponding commercial native form of enzyme; **[b]** corresponding commercial sol-gel immobilized enzyme;[c] sol-gel immobilized non-imprinted [d] sol-gel immobilized bioimprinted with LA; [e] sol-gel immobilized bioimprinted with OA; [f] sol-gel immobilized bioimprinted with TEG; [g] sol-gel immobilized bioimprinted with PEG400; [h] sol-gel immobilized bioimprinted with PEG1000; [i] sol-gel immobilized bioimprinted with BRIJ;[j] sol-gel immobilized bioimprinted with L-TEG-L;[k] sol-gel immobilized bioimprinted with TRX100; [l] sol-gel immobilized bioimprinted with olive oil (Referring to Table 16 in Results and Discussion)



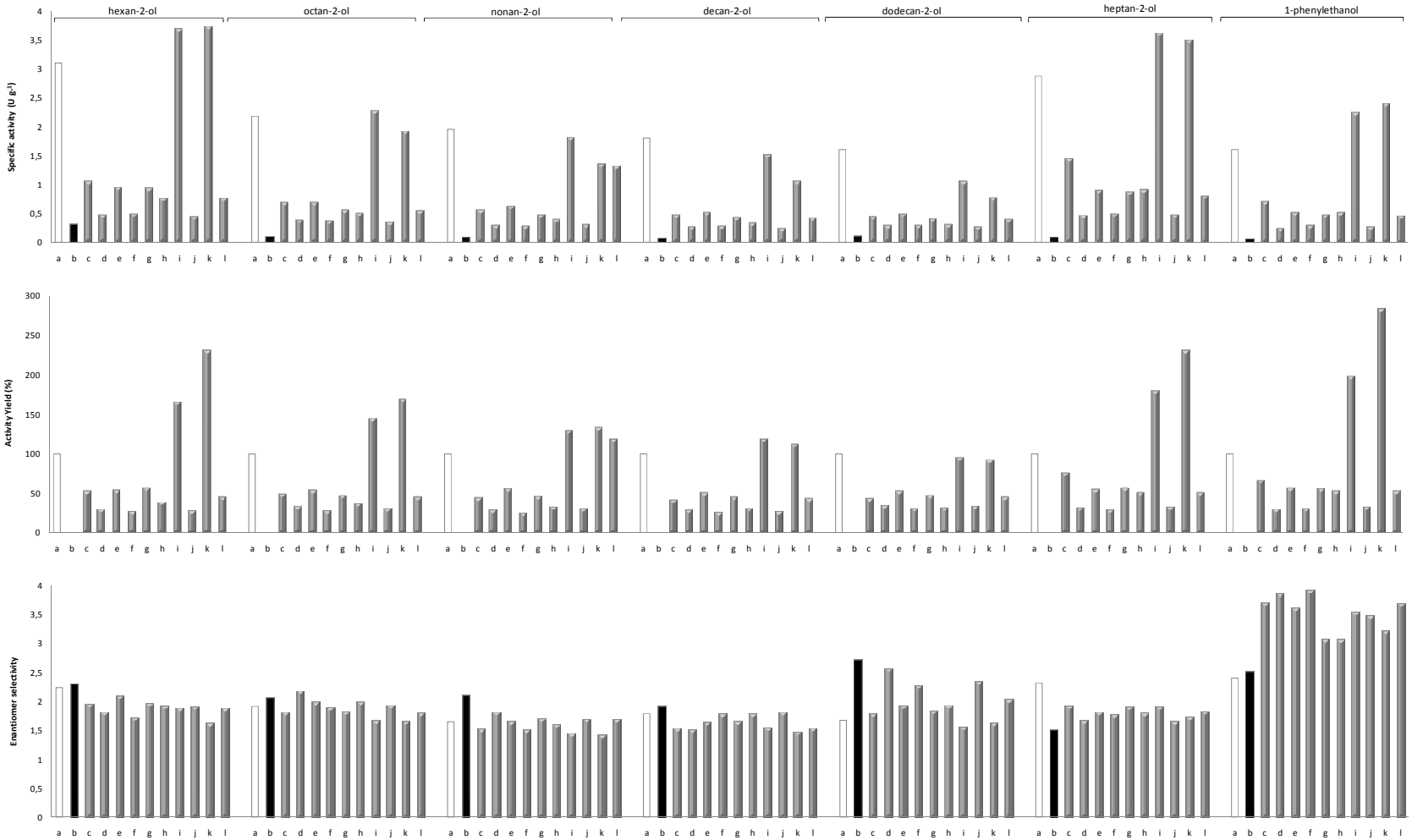
Annex 5. Screening of the additives for bioimprinting in batch mode and characterization of sol-gel immobilized bioimprinted Lipase PS in binary sol-gel systems of TEOS: PhTEOS silane precursors. Specific activity (U_B) activity yield (Y_A)and enantiomer selectivity (E) of the free and immobilized forms Data of LPS represent 2hrs.
[a] corresponding commercial native form of enzyme; **[b]** corresponding commercial sol-gel immobilized enzyme;[c] sol-gel immobilized non-imprinted [d] sol-gel immobilized bioimprinted with LA; [e] sol-gel immobilized bioimprinted with OA; [f] sol-gel immobilized bioimprinted with TEG; [g] sol-gel immobilized bioimprinted with PEG400; [h] sol-gel immobilized bioimprinted with PEG1000; [i] sol-gel immobilized bioimprinted with BRIJ;[j] sol-gel immobilized bioimprinted with L-TEG-L;[k] sol-gel immobilized bioimprinted with TRX100; [l] sol-gel immobilized bioimprinted with olive oil (Referring to Table 16 in Results and Discussion)



Annex 6. Screening of the additives for bioimprinting in batch mode and characterization of sol-gel immobilized bioimprinted lipase CaLB in binary sol-gel systems of TEOS: PhTEOS silane precursors. Specific activity (U_B) activity yield (Y_A)and enantiomer selectivity (E) of the free and immobilized forms Data of CaLB represent 2hrs.
[a] Novozym carrier :liquid CaLB (1:8); **[b]** commercial Novozyme 435;[c] sol-gel immobilized non-imprinted [d] sol-gel immobilized bioimprinted with LA; [e] sol-gel immobilized bioimprinted with OA; [f] sol-gel immobilized bioimprinted with TEG; [g] sol-gel immobilized bioimprinted with PEG400; [h] sol-gel immobilized bioimprinted with PEG1000; [i] sol-gel immobilized bioimprinted with BRIJ;[j] sol-gel immobilized bioimprinted with L-TEG-L;[k] sol-gel immobilized bioimprinted with TRX100; [l] sol-gel immobilized bioimprinted with olive oil (Referring to Table 16 in Results and Discussion)



Annex 7. Screening of the additives for bioimprinting in batch mode and characterization of sol-gel immobilized bioimprinted lipase CaLB in binary sol-gel systems of TEOS: PhTEOS silane precursors. Specific activity (U_B) activity yield (Y_A)and enantiomer selectivity (E) of the free and immobilized forms Data of CaLB represent 2hrs.
[a] Novozym carrier :liquid CaLB (1:8); **[b]** commercial Novozyme 435;[c] sol-gel immobilized non-imprinted [d] sol-gel immobilized bioimprinted with LA; [e] sol-gel immobilized bioimprinted with OA; [f] sol-gel immobilized bioimprinted with TEG; [g] sol-gel immobilized bioimprinted with PEG400; [h] sol-gel immobilized bioimprinted with PEG1000; [i] sol-gel immobilized bioimprinted with BRIJ;[j] sol-gel immobilized bioimprinted with L-TEG-L;[k] sol-gel immobilized bioimprinted with TRX100; [l] sol-gel immobilized bioimprinted with olive oil (Referring to Table 16 in Results and Discussion)



Annex 8. Screening of the additives for bioimprinting in batch mode and characterization of sol-gel immobilized bioimprinted LAK in binary sol-gel systems of TEOS: PhTEOS silane precursors. Specific activity (U_B) activity yield (Y_A)and enantiomer selectivity (E) of the free and immobilized forms Data of LAK represent 2hrs. (Referring to Table 16 in Results and Discussion)

	2a			2b			2c			2d			2e			2f			2g		
	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E
Lipase AK	38.8	79.6	14	49.5	97.7	97.7	44.4	87.7	32	47.8	63.0	7.8	48.3	78.1	18	45.7	87.7	34	39.9	77.1	13
Lipase AK imm	20.3	87.4	18	47.2	99.5	99.5	28.4	95.4	62	26.0	70.2	7.2	28.8	87.1	21	28.1	93.5	43	20.8	82.3	13
Blank	33.2	78.4	12	48.9	97.2	97.2	46.5	84.1	25	48.7	57.4	6.2	47.5	76.8	16	42.6	85.9	25	33.9	74.7	10
LA	29.9	77.6	11	46.5	96.9	96.9	27.8	86.9	20	24.8	54.5	4.0	25.1	79.5	11	21.7	87.5	19	14.8	69.6	6.3
OA	16.8	78.6	9.8	40.1	98.1	98.1	39.7	85.2	22	42.1	55.3	5.1	42.9	75.9	13	38.2	85.5	22	30.8	72.3	8.5
TEG	18.7	77.7	9.5	40.8	97.5	97.5	38.4	85.8	22	36.3	54.9	4.6	36.4	77.5	12	31.1	86.4	20	22.5	71.2	7.3
PEG 400	36.7	78.5	13	49.6	97.1	97.1	40.7	85.1	22	41.0	55.4	5.0	41.5	76.8	13	36.6	86.1	22	28.0	74.1	8.9
PEG 1000	25.7	78.9	11	45.6	97.4	97.4	39.8	86.0	23	42.1	55.9	5.2	43.0	77.5	14	38.9	86.7	24	31.0	73.6	9.0
BRIJ	29.1	76.4	10	46.7	96.8	96.8	28.0	87.9	22	23.7	56.0	4.2	24.1	82.0	13	20.4	89.6	23	13.7	72.6	7.1
L-TEG-L	27.4	77.3	10	45.6	97.0	97.0	11.7	87.8	17	10.4	34.0	2.1	9.0	79.3	9.4	7.5	84.3	13	5.1	55.1	3.6
TRX100	30.8	77.9	11	47.7	97.0	97.0	37.8	83.7	19	36.8	53.3	4.4	36.5	75.6	11	31.7	85.1	18	23.1	71.5	7.4
Olive oil	6.4	77.8	8.5	21.4	97.6	97.6	33.0	85.8	20	32.4	53.9	4.3	33.1	77.0	11	28.3	85.9	18	21.0	70.2	6.9

Multisubstrate „A" system (2c-g) and Multisubstrate „B" system (2a,b).

The *ee* values are determined by enantioselective GC.

The enantiomer selectivity (E) was calculated from *c* $ee_{(R)\text{-}2}$[]. Due to sensitivity to experimental errors, E values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

Annex 9. Screening of the additives for bioimprinting in batch mode and characterization of sol-gel immobilized bioimprinted LPS in binary sol-gel systems of TEOS: PhTEOS silane precursors. Specific activity (U_B) activity yield (Y_A)and enantiomer selectivity (E) of the free and immobilized forms Data of LPS represent 2hrs. (Referring to Table 16 in Results and Discussion)

	2a			2b			2c			2d			2e			2f			2g		
	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E
Lipase PS	5.0	85.2	13	20.7	99.5	>200	6.8	84.7	13	7.0	74.1	7.1	8.0	85.0	13	7.3	83.1	12	5.1	0.7	6.2
Lipase PS imm	5.4	88.6	18	24.0	99.6	>>200	2.5	87.8	16	8.3	79.8	9.5	9.6	85.5	14	8.8	82.9	12	5.5	0.7	7.0
Blank	37.9	83.2	18	49.0	99.1	>>200	48.8	74.2	15	48.7	67.0	10	49.5	73.4	15	48.7	68.6	11	33.7	83.9	18.0
LA	43.0	80.6	18	49.6	98.9	>>200	51.6	73.4	16	52.3	65.5	10	53.4	71.3	15	53.3	66.6	11	38.9	83.7	19.6
OA	28.8	85.0	18	49.0	97.8	>>200	43.3	76.8	15	43.3	67.6	9.2	44.6	76.0	15	43.6	71.8	12	30.0	84.3	17.5
TEG	34.3	83.6	17	49.2	99.3	>>200	43.6	77.3	14	42.0	69.2	9.0	44.0	77.3	14	42.1	73.5	11	27.7	84.5	16.3
PEG 400	44.7	80.8	19	48.7	99.0	>>200	39.6	73.2	13	47.6	70.1	11	50.5	74.3	16	50.5	69.6	12	36.3	85.0	20.2
PEG 1000	34.2	84.4	18	49.0	99.3	>>200	34.7	78.2	13	40.4	70.4	9.2	43.3	78.9	15	42.5	74.7	12	29.1	86.1	19.0
BRIJ	19.0	81.5	12	38.8	99.0	>200	28.5	72.9	8.6	23.9	63.6	5.7	23.2	75.5	9.1	20.5	68.8	6.5	12.1	71.1	6.7
L-TEG-L	37.4	82.7	18	49.4	99.1	>>200	50.3	74.7	16	50.4	67.1	10	51.8	72.9	15	51.0	68.4	11	36.2	84.6	19.2
TRX100	7.6	82.0	11	26.0	99.3	>200	14.8	76.3	8.6	12.1	66.9	5.7	12.4	84.4	14	10.7	80.5	10	6.7	70.9	6.9
Olive oil	19.9	84.7	15	40.9	99.3	>>200	24.8	80.9	12	23.7	67.5	6.4	24.3	79.6	12	21.3	78.8	10	12.7	81.0	10.9

Multisubstrate „A" system (2c-g) and Multisubstrate „B" system (2a,b).

The *ee* values are determined by enantioselective GC.

The enantiomer selectivity (E) was calculated from *c* $ee_{(R)\text{-}2}$[(CHEN C. S., 1982)]. Due to sensitivity to experimental errors, E values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

Annex 10. Screening of the additives for bioimprinting in batch mode and characterization of sol-gel immobilized bioimprinted CaLB in binary sol-gel systems of TEOS: PhTEOS silane precursors. Specific activity (U_B) activity yield (Y_A)and enantiomer selectivity (E) of the free and immobilized forms Data of CaLB represent 2hrs. (Referring to Table 16 in Results and Discussion)

	2a			2b			2c			2d			2e			2f			2g		
	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E
1:8 ratio	49.4	90.3	58	48.5	99.1	>>200	52.1	88.3	63	50.7	93.6	>100	52.0	94.2	>>200	50.5	95.2	>100	50.1	95.9	>100
Novozyme 435	51.7	91.2	98	49.0	99.5	>>200	50.6	89.9	62	50.4	92.8	96	51.2	93.3	>100	51.0	94.6	>100	50.0	94.4	>100
Blank	39.9	99.2	>200	25.5	99.2	>200	32.5	99.8	>>200	19.9	99.8	>>200	16.5	98.2	>100	15.4	98.8	>100	13.6	93.3	34
LA	37.3	99.3	>200	22.8	99.5	>>200	37.9	99.2	>200	23.3	99.4	>200	19.2	98.9	>200	18.1	99.1	>200	15.5	93.3	34
OA	35.9	99.2	>200	29.5	99.7	>>200	47.9	99.0	>>200	31.4	99.0	>200	22.9	98.8	>200	17.5	99.0	>200	10.6	93.4	33
TEG	27.6	98.9	>200	12.9	99.5	>200	34.9	99.0	>200	21.3	98.8	>200	17.7	98.7	>100	17.0	99.5	>200	15.4	94.1	39
PEG 400	38.9	99.6	>>200	31.2	99.2	>200	44.8	98.6	>200	25.6	98.9	>200	17.5	97.4	93	11.7	98.4	>100	5.0	83.8	12
PEG 1000	34.3	99.5	>>200	20.7	99.4	>200	44.9	99.3	>>200	34.0	99.1	>200	30.3	98.9	>200	28.1	99.3	>200	22.6	96.8	81
BRIJ	44.6	98.7	>200	38.6	99.6	>>200	48.5	98.4	>200	47.1	99.0	>>200	45.3	98.6	>200	43.3	99.5	>>200	39.1	97.7	>100
L-TEG-L	32.5	99.2	>200	16.5	99.2	>200	43.0	99.3	>>200	36.1	98.9	>200	32.6	99.0	>200	31.4	99.4	>>200	26.8	97.3	>100
TRX100	42.4	98.6	>200	26.8	99.4	>>200	48.3	98.8	>>200	44.5	98.9	>200	42.0	98.9	>200	41.5	99.2	>>200	38.1	97.7	>100
Olive oil	38.2	99.2	>200	23.1	99.5	>>200	46.2	99.4	>>200	38.5	99.1	>200	34.6	98.9	>200	32.8	99.2	>200	27.6	97.4	>100

Multisubstrate „A" system (2c-g) and Multisubstrate „B" system (2a,b).
The *ee* values are determined by enantioselective GC.
The enantiomer selectivity (E) was calculated from *c* $ee_{(R)\text{-}\mathbf{2}}$ [(CHEN C. S., 1982)]. Due to sensitivity to experimental errors, E values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

Annex 11. Screening of the additives for bioimprinting in batch mode and characterization of sol-gel immobilized bioimprinted CrL in binary sol-gel systems of TEOS: PhTEOS silane precursors. Specific activity (U_B) activity yield (Y_A)and enantiomer selectivity (E) of the free and immobilized forms Data of CrL represent 8hrs. (Referring to Table 16 in Results and Discussion)

	2a			2b			2c			2d			2e			2f			2e		
	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E	c (%)	ee (%)	E
CcL	18.0	36.4	2.3	9.7	39.5	2.4	26.1	33.4	2.2	18.4	28.4	1.9	16.4	22.4	1.6	15.1	26.1	1.8	13.5	23.4	1.7
CcL imm	0.5	19.6	1.5	0.3	42.8	2.5	0.9	34.5	2.3	0.8	34.5	2.1	0.7	35.4	2.1	0.6	31.0	1.9	0.9	46.0	2.7
Blank	4.5	30.6	1.9	2.2	56.4	3.7	4.4	31.5	2.0	2.9	28.0	1.8	2.3	19.4	1.5	2.0	20.3	1.5	1.9	27.3	1.8
LA	1.4	23.2	1.7	0.8	57.3	3.8	2.0	28.0	1.8	1.6	35.7	2.2	1.2	28.4	1.8	1.1	19.7	1.5	1.2	42.3	2.5
OA	2.8	27.9	1.8	1.6	55.3	3.6	4.0	34.5	2.1	2.8	32.5	2.0	2.6	23.8	1.6	2.2	23.6	1.6	2.0	31.0	1.9
TEG	1.5	26.7	1.8	0.9	58.2	3.9	2.1	24.9	1.7	1.5	30.0	1.9	1.2	19.1	1.5	1.2	26.2	1.8	1.2	37.8	2.3
PEG 400	2.7	30.3	1.9	1.5	50.4	3.1	3.9	31.8	2.0	2.3	28.2	1.8	2.0	25.4	1.7	1.8	24.2	1.6	1.7	28.8	1.8
PEG 1000	2.8	26.9	1.8	1.6	50.3	3.1	3.2	30.0	1.9	2.1	32.2	2.0	1.7	22.4	1.6	1.4	27.3	1.8	1.3	31.5	1.9
BRIJ	11.3	29.2	1.9	7.0	54.4	3.5	15.5	27.9	1.9	9.5	23.5	1.7	7.6	16.8	1.4	6.3	20.5	1.5	4.4	20.7	1.5
L-TEG-L	1.5	22.7	1.6	0.8	54.4	3.5	1.9	29.9	1.9	1.4	30.8	1.9	1.3	24.8	1.7	1.0	28.3	1.8	1.1	39.1	2.3
TRX100	10.9	25.1	1.7	7.5	50.8	3.2	15.7	21.7	1.6	8.1	23.3	1.6	5.7	16.4	1.4	4.4	18.1	1.5	3.2	22.8	1.6
Olive oil	2.5	27.9	1.8	1.4	56.7	3.7	3.2	29.8	1.9	2.3	27.7	1.8	5.5	24.5	1.7	1.8	20.3	1.5	1.6	33.4	2.0

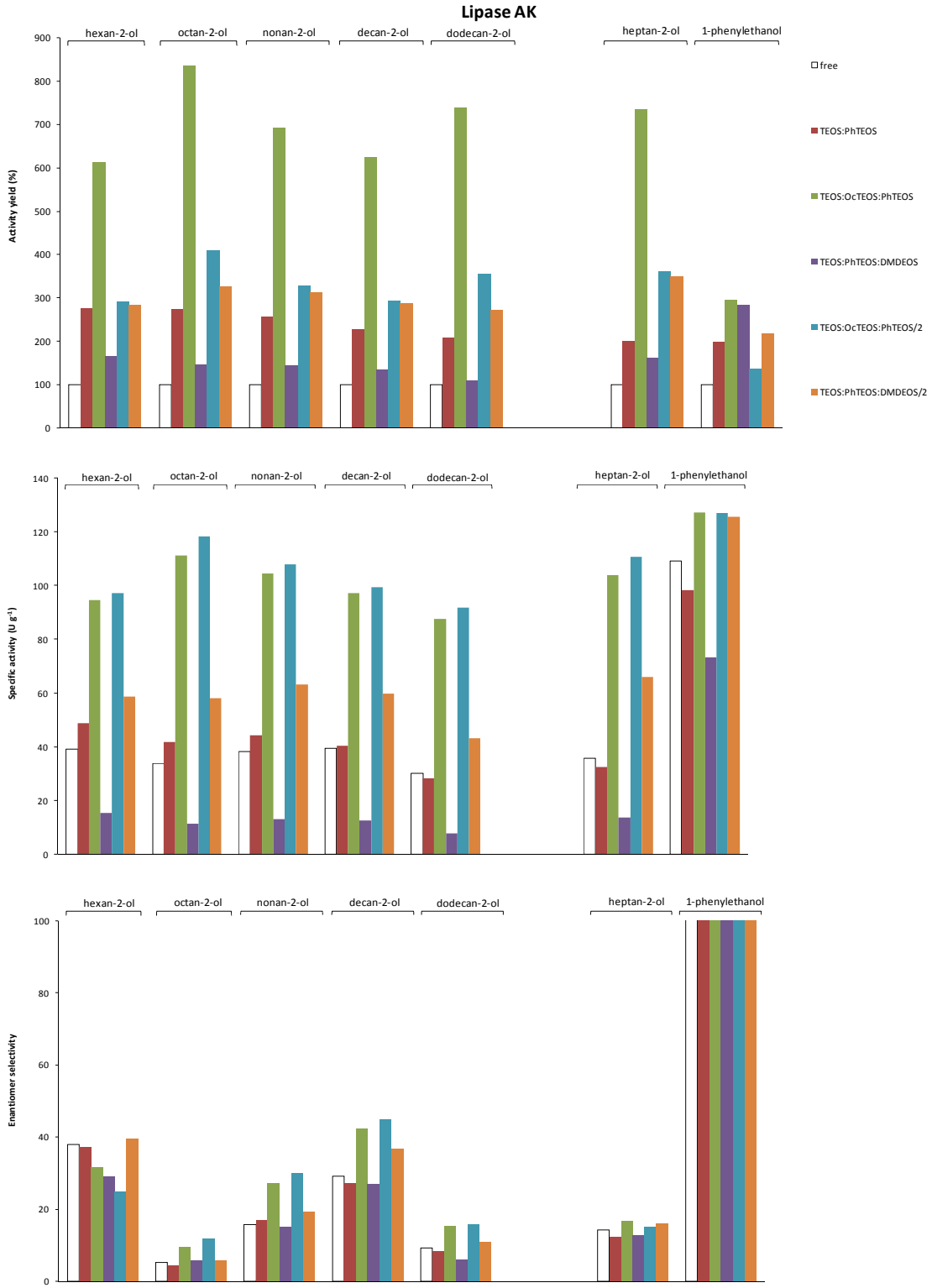
Multisubstrate „A" system (2c-g) and Multisubstrate „B" system (2a,b).
The *ee* values are determined by enantioselective GC.
The enantiomer selectivity (E) was calculated from *c* $ee_{(R)\text{-}\mathbf{2}}$ [(CHEN C. S., 1982)]. Due to sensitivity to experimental errors, E values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as >200 and values calculated above 500 are given as »200.

Annex 12. Comparison of the best performing sol-gel entrapped and bioimprinted lipase AK in biner and terner silane matrices in batch mode (in hexane:THF; 2:1). Specific activity (U_B), activity yield (*YA*) and enantiomer selectivity (E) of the free forms of lipases Data of Lipase AK,represent 0.5h results

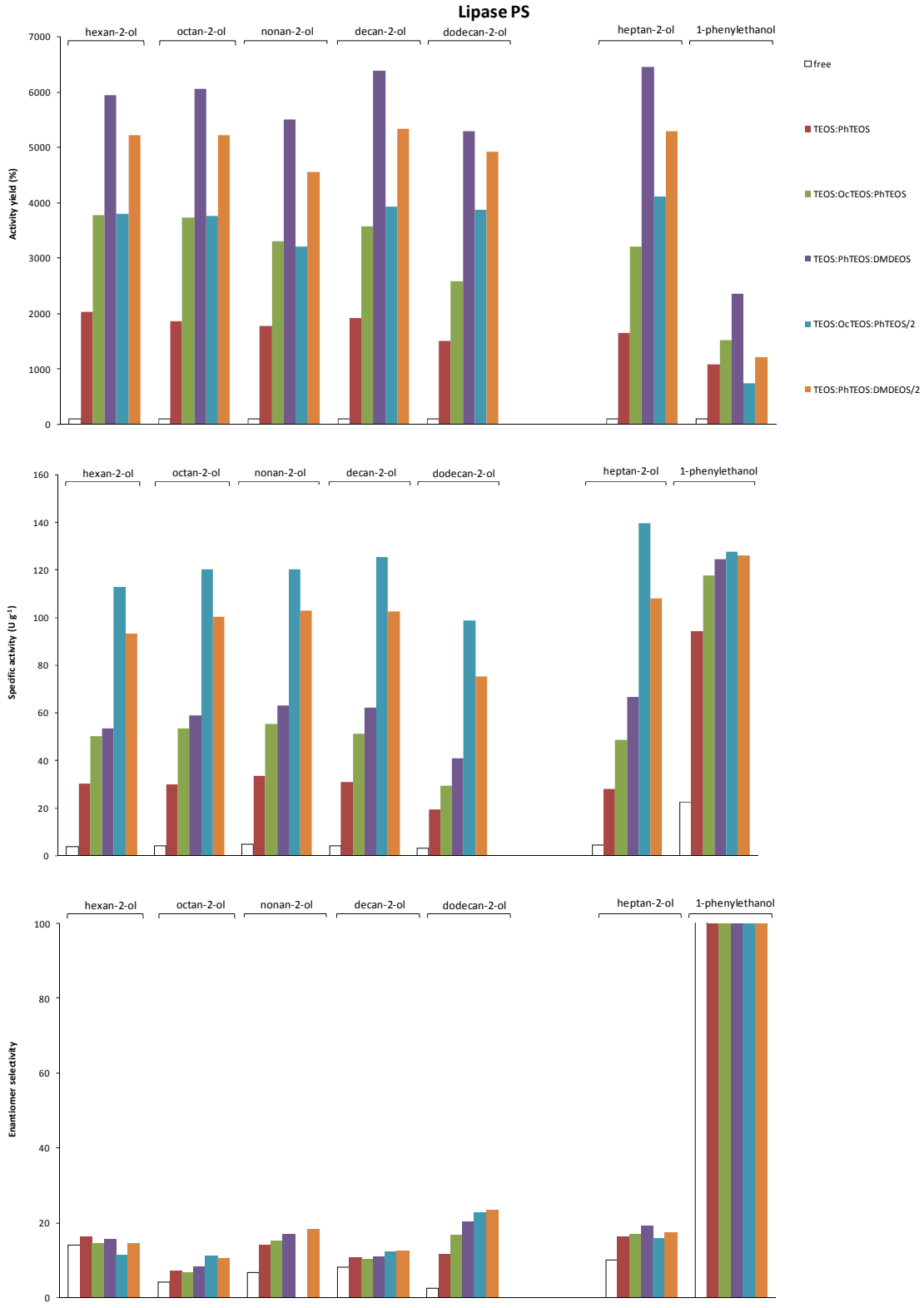
TEOS was kept in constant while alkylTEOS and dialkylTEOS were varied in the precursor systems TEOS:PhTEOS (1:1) TEOS:OcTEOS:PhTEOS (1:0:7:0.3), TEOS:OCTEOS:DMDEOS (4:1:1)

Normal (single) enzyme loading (see it detailed in experimental part)

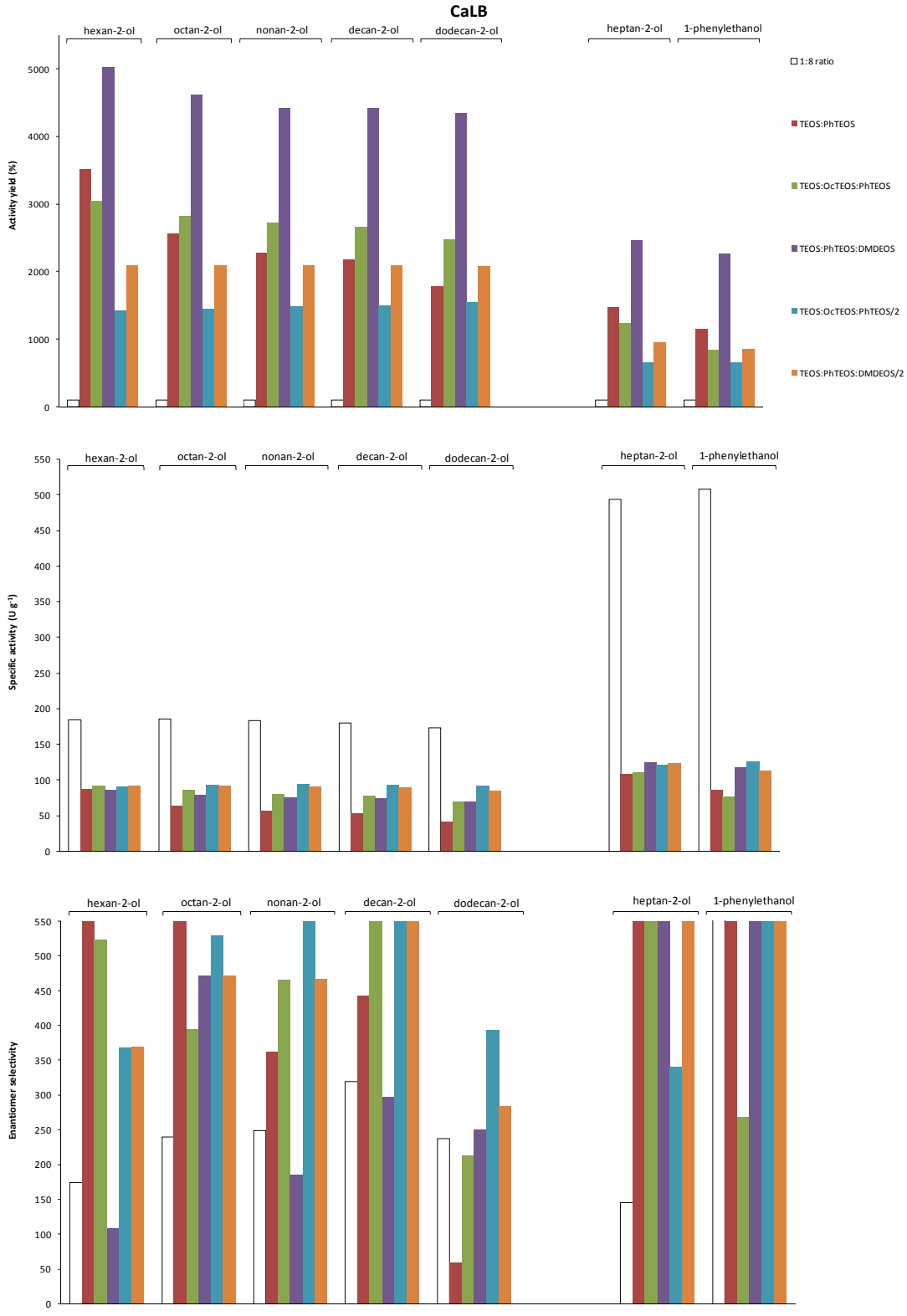
[/2] Enzyme loading was double than normal (Referring to Table 17 in Results and Discussion)



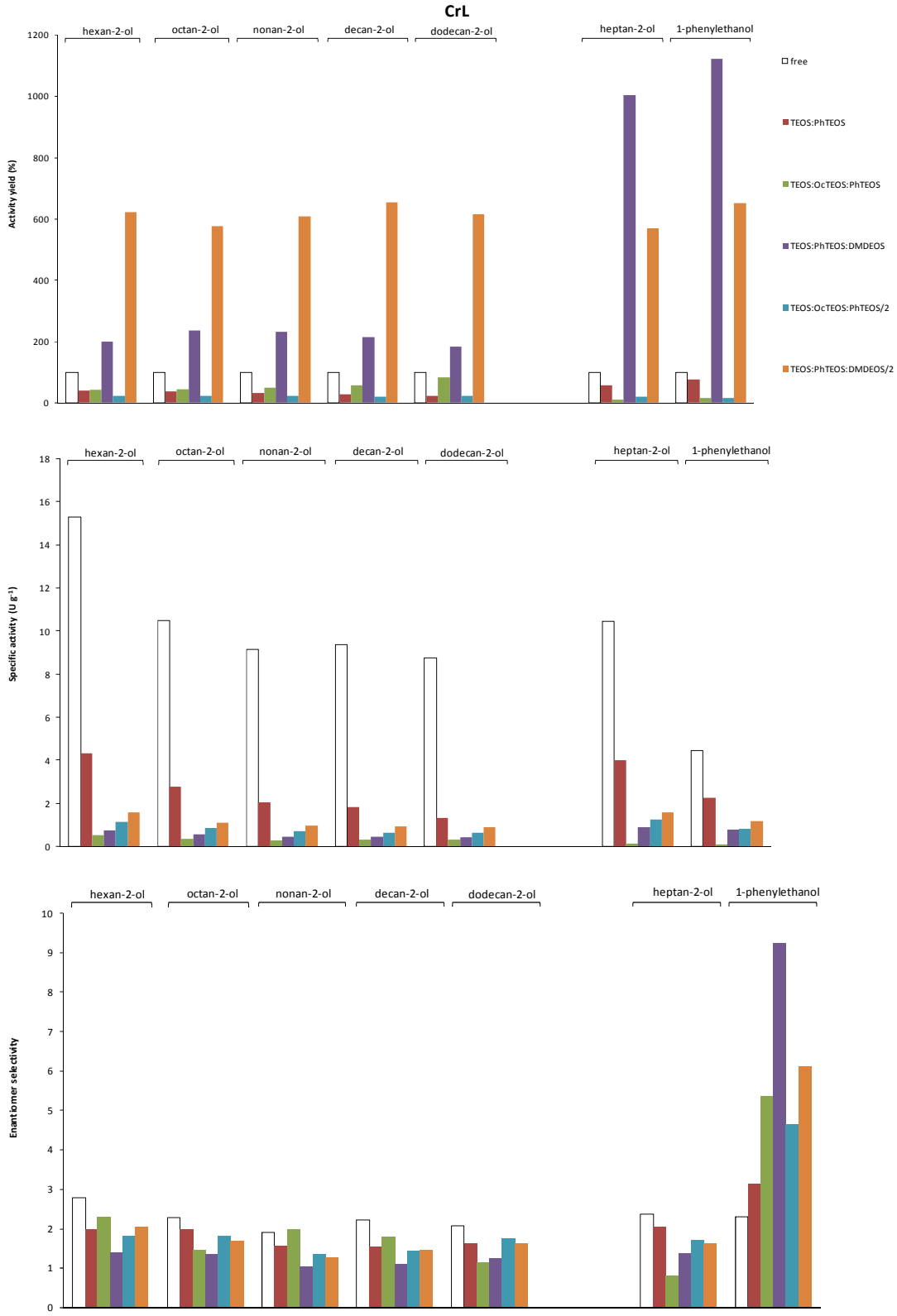
Annex 13. Comparison of the best performing sol-gel entrapped and bioimprinted lipase PS in biner and terner silane matrices in batch mode (in hexane:THF; 2:1). Specific activity (U_B), activity yield (*YA*) and enantiomer selectivity (E) of the free forms of lipases Data of Lipase PS,represent 0.5h results (Referring to Table 17 in Results and Discussion)



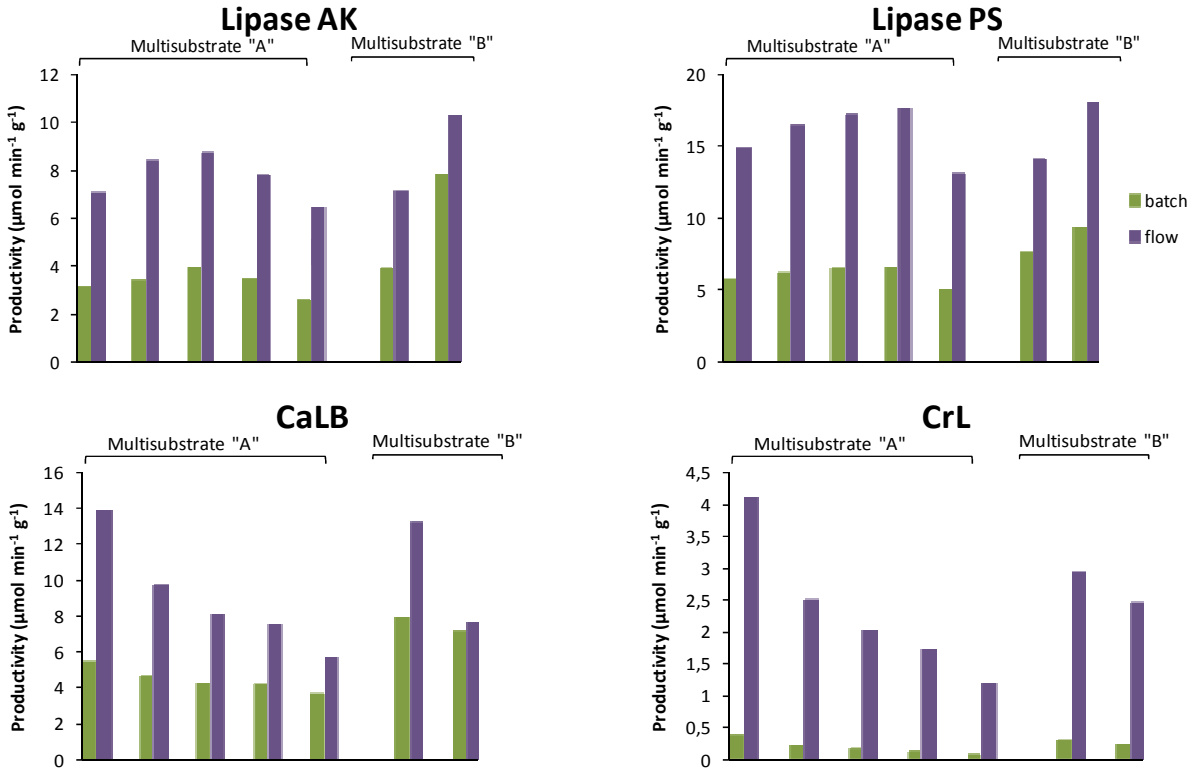
Annex 14. Comparison of the best performing sol-gel entrapped and bioimprinted CaLB in biner and terner silane matrices in batch mode (in hexane:THF; 2:1). Specific activity (U_B), activity yield (*YA*) and enantiomer selectivity (E) of the free forms of lipases Data of Lipase PS,represent 0.5h results (Referring to Table 17 in Results and Discussion)



Annex 15. Comparison of the best performing sol-gel entrapped and bioimprinted lipase CrL in biner and terner silane matrices in batch mode (in hexane:THF; 2:1). Specific activity (U_B), activity yield (*YA*) and enantiomer selectivity (E) of the free forms of lipases Data of Lipase PS,represent 4h results (Referring to Table 17 in Results and Discussion)



Annex 16. Comparison of the best performing sol-gel entrapped and bioimprinted lipases' productivity (specific reaction rate"*r*") tested in multisubstrate systems („A" *rac*-**1c-g** and „B", *rac*-**1a,b**) of batch and continuous-flow packed-bed reactor (in hexane: TMBE; 2:1). (Referring to Figure 27 in Results and Discussion)



KÖSZÖNETNYILVÁNÍTÁS

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