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DESIGN OF NEW IMMOBILIZED LIPASES FOR BIOTRANSFORMATIONS IN AQUEOUS AND ORGANIC MEDIA

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Doctoral thesis presented as part of the requirements to obtain the title of Doctor in Chemical Engineering by the Federal University of São Carlos (UFSCar), in the field of Research and Development of Chemical Processes.

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Start before you're ready.

ABSTRACT

DESIGN OF NEW IMMOBILIZED LIPASES FOR BIOTRANSFORMATIONS IN AQUEOUS AND ORGANIC MEDIA - A review of the literature covering research on the immobilization of lipases on hydrophobic supports was performed using systematic mapping (MS) concepts. The MS approach enabled the identification of gaps that led to the development of this thesis. The mineralization of lipases immobilized with metal phosphate was the main focus of the study. This strategy is an alternative to solve the nanoflowers fragility while maintaining some of the mineralization benefits. When mineralization is performed on previously immobilized enzymes, the researcher can select the support based on its mechanical resistance, avoiding the difficulties derived from the management of the small and fragile nanoflowers. Moreover, the mineralization of immobilized enzyme couples the positive effects of enzyme mineralization during nanoflowers production with the benefits of enzyme immobilization in preexisting solids. Several lipases were immobilized on octyl agarose beads via interfacial activation and modified with diverse metal phosphates. It was found that the effects of the metal phosphate modification were clearer and more positive using highly loaded biocatalyst, suggesting that enzyme crowding could facilitate some of the positive effects of enzyme mineralization. The effects depended on the nature of both enzyme and metallic phosphate. The incubation with only sodium phosphate or only metal chloride, as well as the immobilization on previously modified supports which produced significantly reduced effects. The immobilized enzyme mineralization cannot produce a tridimensional nanoflower, as the enzymes will be located on a flat planar surface, but the results suggest that the positive effects of the building of nanoflowers may be, at least partially, achieved using this solid-phase strategy. However, we cannot talk of nanoflowers, as these tridimensional structures will never be achieved. The study was later extended to the use of diverse commercial biocatalysts and *Thermomyces lanuginosus* lipase (TLL) immobilized on Purolite@ C18. The modifications greatly altered enzyme specificity, increasing the activity versus some substrates while decreasing the activity versus other substrates. Enantiospecificity was also drastically altered after these modifications. Regarding the enzyme stability, no significant positive effects were found; in fact, a decrease in enzyme stability was usually detected.

The influence of the immobilization protocol on the effects of mineralization was investigated. The stability, activity and specificity of the biocatalysts were very different, both the differently blocked vinyl sulfone biocatalysts (VS-biocatalysts) and the glutaraldehyde

biocatalysts prepared at different pH. The activity, specificity and stability effects of the mineralization strongly depended on the enzyme and on the immobilization protocol. For the same enzyme, a mineralization protocol could be negative, positive or present no effect depending on the enzyme immobilization procedure and substrate. These results highlight the great potential of mineralization of immobilized enzymes to improve their properties, as well as the great interactions that immobilization protocol and mineralization can exhibit. The combination of both methodologies greatly increases the possibilities to find a biocatalyst that can be suitable for a specific process. The mineralization of chemically or physically modified immobilized lipases is also a potent tool to improve enzyme features. The changes caused by chemical modifications with glutaraldehyde, trinitrobenzenesulfonic acid or ethylenediamine and carbodiimide, or physical coating with ionic polymers, such as polyethylenimine and dextran sulfate have, in most cases, negative effects with some substrates and positive with other ones. Furthermore, the same mineralization could present different effects on the enzyme activity, specificity or stability, depending on the previous modification performed on the enzyme, showing that these previous enzyme modifications alter the effects of the mineralization on enzyme features. In this way, the combination of chemical or physical modifications of enzymes before their mineralization increases the range of modification of features that the immobilized enzyme can experienced, enabling to enlarge the biocatalyst library.

Eversa$^{@}$ Transform immobilized on Purolite$^{@}$ C18 was successfully applied in the esterification of purified fatty acids of the hydrolysis of degummed soybean oil for the synthesis of octyl esters. Furthermore, aiming at the application of biocatalysts in organic reactions, TLL was immobilized on Purolite$^{@}$ C18 aminated and activated with vinyl sulfone. The use of different blocking agents as reaction end point (using ethylene-diamine, aspartic acid, glycine, and cysteine) greatly altered the biocatalyst functional features (activity, specificity, or stability). Furthermore, the differently blocked VS-biocatalysts showed different performances in the synthesis of fatty acid methyl esters. In general, they showed better affinity for the transesterification of polyunsaturated oils.

Keywords: solid-phase enzyme mineralization, enzyme stabilization, tuning enzyme specificity, immobilized lipase physicochemical modification, heterofunctional supports.

RESUMO

DESIGN DE NOVAS LIPASES IMOBILIZADAS PARA BIOTRANSFORMAÇÕES EM MEIOS AQUOSO E ORGÂNICO - Uma revisão da literatura sobre imobilização de lipases em suportes hidrofóbicos foi realizada usando conceitos de mapeamento sistemático (MS). A abordagem de MS possibilitou a identificação de lacunas que levaram ao desenvolvimento desta tese. A mineralização de lipases imobilizadas com fosfato metálico foi o principal foco de estudo. Essa estratégia é uma alternativa para resolver a fragilidade das nanoflores, mantendo alguns dos benefícios da mineralização. Quando a mineralização for realizada sobre enzimas previamente imobilizadas, o pesquisador pode selecionar o suporte com base em sua resistência mecânica, evitando as dificuldades decorrentes do manejo das pequenas e frágeis nanoflores. Além disso, a mineralização da enzima imobilizada combina os efeitos positivos da mineralização da enzima durante a produção de nanoflores com os benefícios da imobilização da enzima em sólidos preexistentes. Várias lipases foram imobilizadas em grânulos de octil agarose via ativação interfacial e modificadas com diversos fosfatos metálicos. Verificou-se que os efeitos da modificação do fosfato metálico foram mais claros e mais positivos usando biocatalisador altamente carregado, sugerindo que a aglomeração enzimática poderia facilitar alguns dos efeitos positivos da mineralização. Os efeitos dependeram da natureza da enzima e do fosfato metálico. A incubação apenas com fosfato de sódio ou apenas cloreto de metal, bem como a imobilização em suportes previamente modificados produziram efeitos significativamente reduzidos. A mineralização da enzima imobilizada não pode produzir uma nanoflores tridimensional, pois as enzimas estão localizadas em uma superfície plana, mas os resultados sugerem que os efeitos positivos da construção de nanoflores podem ser alcançados usando esta estratégia em fase sólida. Esse estudo foi posteriormente estendido para o uso de diversos biocatalisadores comerciais e lipase de *Thermomyces lanuginosus* (TLL) imobilizados em Purolite@ C18. As modificações alteraram bastante a especificidade da enzima, aumentando a atividade em relação a alguns substratos e diminuindo a atividade em relação a outros substratos. A enantioespecificidade foi drasticamente alterada após essas modificações. Com relação à estabilidade da enzima, não foram encontrados efeitos positivos significativos.

A influência do protocolo de imobilização sobre os efeitos da mineralização foi investigada. A estabilidade, atividade e especificidade dos biocatalisadores foram muito diferentes, tanto os vinil-sulfona-biocatalisadores (VS-biocatalisadores) bloqueados de forma diferente quanto os biocatalisadores de glutaraldeído preparados em diferentes pH. Os efeitos

da mineralização sobre a atividade, especificidade e estabilidade dependeram fortemente da enzima e do protocolo de imobilização. Para uma mesma enzima, um protocolo de mineralização pode ser negativo, positivo ou não apresentar efeito dependendo do procedimento de imobilização da enzima e do substrato. Esses resultados destacam o grande potencial de mineralização das enzimas imobilizadas para melhorar suas propriedades, bem como as grandes interações que o protocolo de imobilização e a mineralização podem apresentar. A combinação das duas metodologias aumenta muito as possibilidades de encontrar um biocatalisador que possa ser adequado para um processo específico. A mineralização de lipases imobilizadas quimicamente ou fisicamente modificadas também é uma ferramenta potente para melhorar as características da enzima. As alterações causadas por modificações químicas com glutaraldeído, ácido trinitrobenzenossulfônico ou etilenodiamina e carbodiimida, ou revestimento físico com polímeros iônicos, como polietilenonimina e sulfato de dextrana têm, na maioria das vezes, efeitos negativos com alguns substratos e positivos com outros. Além disso, uma mesma mineralização pode apresentar diferentes efeitos sobre a atividade, especificidade ou estabilidade da enzima, dependendo da modificação anterior realizada na enzima, mostrando que essas modificações enzimáticas anteriores alteram os efeitos da mineralização nas características da enzima. Desta forma, a combinação de modificações químicas ou físicas de enzimas antes de sua mineralização aumenta a gama de modificações de características que a enzima imobilizada pode experimentar, permitindo ampliar a biblioteca de biocatalisadores.

Eversa$^{@}$ Transform imobilizada em Purolite$^{@}$ C18 foi aplicada com sucesso na esterificação de ácidos graxos purificados da hidrólise de óleo de soja degomado para a síntese de ésteres octílicos. Além disso, visando a aplicação de biocatalisadores em reações orgânicas, TLL foi imobilizado em Purolite$^{@}$ C18 aminado e ativado com vinil sulfona. O uso de diferentes agentes bloqueadores como ponto final da reação (usando etileno-diamina, ácido aspártico, glicina e cisteína) alterou bastante as características funcionais do biocatalisador (atividade, especificidade ou estabilidade). Além disso, os VS-biocatalisadores bloqueados de forma diferente mostraram desempenhos diferentes na síntese de ésteres metílicos de ácidos graxos. Em geral, apresentaram melhor afinidade para a transesterificação de óleos poliinsaturados.

Palavras-chave: mineralização enzimática em fase sólida, estabilização enzimática, ajuste da especificidade enzimática, modificação físico-química de lipase imobilizada, suportes heterofuncionais.

RESUMEN

DISEÑO DE NUEVAS LIPASAS INMOVILIZADAS PARA BIOTRANSFORMACIONES EN MEDIOS ACUOSO Y ORGÁNICO - Una revisión de la literatura sobre inmovilización de lipasas en soportes hidrofóbicos fue realizada usando conceptos de mapeo sistemático (MS). El enfoque de la MS permitió la identificación de las lagunas que llevaron al desarrollo de esta tesis. La mineralización de lipasas inmovilizadas con fosfato metálico fue el principal foco de estudio. Esta estrategia es una alternativa para resolver la fragilidad de las nanoflowers, manteniendo algunos de los beneficios de la mineralización. Cuando la mineralización se realiza sobre enzimas previamente inmovilizadas, el investigador puede seleccionar el soporte con base en su resistencia mecánica evitando las dificultades derivadas del manejo de las pequeñas y frágiles nanoflowers. Además, la mineralización de la enzima inmovilizada combina los efectos positivos de la mineralización de la enzima durante la producción de nanoflowers con los beneficios de la inmovilización de la enzima en sólidos preexistentes. Varias lipasas fueron inmovilizadas en gránulos de octil agarosa vía activación interfacial y modificadas con diversos fosfatos metálicos. Se constató que los efectos de la modificación del fosfato metálico fueron más claros y más positivos usando biocatalizador altamente cargado, sugiriendo que la aglomeración enzimática podría facilitar algunos de los efectos positivos de la mineralización. Los efectos dependieron de la naturaleza de la enzima y del fosfato metálico. La incubación solo con fosfato de sodio o solo cloruro de metal, así como la inmovilización en soportes previamente modificados han producido efectos significativamente reducidos. La mineralización de la enzima inmovilizada no puede producir una nanoflower tridimensional, pues las enzimas están localizadas en una superficie plana, pero los resultados sugieren que los efectos positivos de la construcción de nanoflowers pueden ser logrados utilizando esta estrategia en fase sólida. Este estudio fue extendido para el uso de diversos biocatalizadores comerciales y lipasa de *Thermomyces lanuginosus* (TLL) inmovilizados en Purolite@ C18. Las modificaciones alteraron bastante la especificidad de la enzima, aumentando la actividad en relación a algunos sustratos y disminuyendo la actividad en relación a otros sustratos. La enantioespecificidad se ha alterado drásticamente después de estas modificaciones. Con respecto a la estabilidad de la enzima, no se encontraron efectos positivos significativos.

Se investigó la influencia del protocolo de inmovilización sobre los efectos de la mineralización. La estabilidad, actividad y especificidad de los biocatalizadores fueron muy diferentes, tanto los VS-biocatalizadores bloqueados de forma diferente como los biocatalizadores de glutaraldehído preparados en diferentes pH. Los efectos de actividad,

especificidad y estabilidad de la mineralización dependieron en gran medida de la enzima y del protocolo de inmovilización. Para una misma enzima, un protocolo de mineralización puede ser negativo, positivo o no presentar efecto dependiendo del procedimiento de inmovilización de la enzima y del sustrato. Estos resultados destacan el gran potencial de mineralización de las enzimas inmovilizadas para mejorar sus propiedades, así como las grandes interacciones que el protocolo de inmovilización y la mineralización pueden presentar. La combinación de ambas metodologías aumenta las posibilidades de encontrar un biocatalizador que pueda ser adecuado para un proceso específico. La mineralización de lipasas inmovilizadas químicamente o físicamente modificadas también es una herramienta potente para mejorar las características de la enzima. Las alteraciones causadas por modificaciones químicas con glutaraldehído, ácido trinitrobencenosulfónico o etilendiamina y carbodiimida, o recubrimiento físico con polímeros iónicos, como polietilenonimina y sulfato de dextrana tienen efectos negativos con algunos sustratos y positivos con otros. Además, una misma mineralización puede presentar diferentes efectos sobre la actividad, especificidad o estabilidad de la enzima, dependiendo de la modificación anterior realizada en la enzima, mostrando que esas modificaciones enzimáticas anteriores alteran los efectos de la mineralización en las características de la enzima. De esta forma, la combinación de modificaciones químicas o físicas de enzimas antes de su mineralización aumenta la gama de modificaciones de características que la enzima inmovilizada puede experimentar, permitiendo ampliar la biblioteca de biocatalizadores.

Eversa$^{@}$ Transform inmovilizada en Purolite$^{@}$ C18 fue aplicada con éxito en la esterificación de ácidos grasos purificados de la hidrólisis de aceite de soja desgomado para la síntesis de ésteres octílicos. Además, para la aplicación de biocatalizadores en reacciones orgánicas, TLL fue inmovilizado en Purolite$^{@}$ C18 aminado y activado con vinilo sulfónico. El uso de diferentes agentes bloqueantes como punto final de la reacción (usando etileno-diamina, ácido aspártico, glicina y cisteína) alteró bastante las características funcionales del biocatalizador (actividad, especificidad o estabilidad). Además, los VS-biocatalizadores bloqueados de manera diferente mostraron diferentes prestaciones en la síntesis de ésteres metílicos de ácidos grasos. En general, presentaron mejor afinidad para la transesterificación de aceites poliinsaturados.

Palabras-clave: mineralización enzimática en fase sólida, estabilización enzimática, ajuste de la especificidad enzimática, modificación físico-química de lipasa inmovilizada, soportes heterofuncionales.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ANL	*Aspergillus niger* lipase
AOL	*Aspergillus oryzae* lipase
ASL	Alcaligenes sp. lipase
ATR-FTIR	Fourier transform infrared spectroscopy operating with attenuated total reflection
BCL	*Burkholderia cepacia* lipase
Bent-CTAB	Na-bentonite-modified with cetyltrimethyl ammonium bromide
BSA	Bovine serum albumin
BTL2	*Geobacillus thermocatenulatus* lipase
BTT	Butyl-Toyopearl
C14	Hydrocarbon chain with 14 carbons
C18	Hydrocarbon chain with 18 carbons
C8	Hydrocarbon chain with 8 carbons
CALA	Lipase A from *Candida antarctica*
CALB	Lipase B from *Candida antarctica*
CCL	*Candida cylindracea* lipase
CEE	Collaboration for Environmental Evidence
CRL	*Candida rugosa* lipase
CSL	*Candida* sp. 99-125 lipase
D3520	Polystyrene-based hydrophobic resin
DA	Dextran aldehyde
Diaion HP-20	Polystyrene-divinylbenzene resin
Diaion HP2-MG	Poly-methacrylate resin
DS	Dextran sulfate
DSO	Degummed soybean oil
DVS	Divinyl-sulfone
EC-OD	EC-octadecyl Sepabeads
ECD	N-3-(Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride
EDA	Ethylenediamine
EV	Eversa Transform 2.0

FAAE	Fatty acid alkyl esters
FAME	Fatty acid methyl ester
FID	Flame ionization detector
G	Graphene
GA	Glutaraldehyde
GC	Gas chromatography
GCLI	*Geotrichum candidum* lipase
GO	Oxidized graphene
HPL	Lipase from Hog Pancreas
HPLC	High Performance Liquid Cromatography
HYPL	*Hypocrea pseudokoningii* lipase
L435	Immobilized *Candida antarctica* Lipase B (Lipozyme®435)
Lifetech ER1061M	Styrene/methacrylic polymer
LipC12	Metagenomic lipase
LS-IM	Immobilized *Rhizomucor miehei* lipase (LipuraSelect)
LU	Lecitase Ultra
MCI gel CHP20P	Styrene-divinylbenzene
MJL	*Mucor javanicus* lipase
MNPS	Octyl-functionalized magnetic nanoparticles
MOF	Metal-organic framework
OC	Octyl-agarose or octyl-sepharose
OCDVS	Divinyl sulfone-activated octyl sepharose
OCEPX	Octyl-epoxide Sepharose
OCGLX	Octyl-glyoxyl Sepharose
ODS	Silica modified with octadecyl
OS	Octyl silica
OTL	*Oncorhynchus tshawytscha* lipase
p-NP	*p*-nitrophenol
p-NPB	*p*-nitrophenyl-butyrate
P(S-co-DVB-co-GMA)	Poly(styrene-co-divinylbenzene-co-glycidyl methacrylate)
P(S-co-DVB)	Poly(styrene-co-divinylbenzene)
PCL	*Pseudomonas cepacian* lipase
PDMS	Polydimethylsiloxane-coated UiO-66

PE	Polyethylene
PEI	polyethyleneimine
PFL	*Pseudomonas fluorescens* lipase
pH	Potential of hydrogen
PHB	Poly-hydroxybutyrate particles
PMA	Poly-methacrylate particles
PMA-co-DVB	Poly(methacrylate-co-divinyl benzene)
PMMA-co-DVB/PMMA-co-DVB	Poly(methyl methacrylate-co-divinylbenzene)/poly(methyl methacrylate-co-divinylbenzene)
PMMA/PMMA	Poly(methyl methacrylate)/poly(methyl methacrylate)
PPL	Porcine pancreas lipase
PS-co-DVB/OS-co-DVB	Poly(styrene-co-divinylbenzene)/poly(styrene-co-divinylbenzene)
PSL	*Pseudomonas stutzeri* lipase
RAL	*Rhizopus arrhizus* lipase
Raw-MWCNT	Multi walled carbon nanotubes
RHS	Rice husk silica
RML	*Rhizomucor miehei* lipase
RML-IM	Immobilized *Rhizomucor miehei* lipase (Lipozyme®RM)
ROL	*Rhizopous oryzae* lipase
RROL	Recombinant *Rhizopous oryzae* lipase
SBA-15	Santa Barbara Amorphous-15
SBA-15@OA	Oleic acid-modified SBA-15
SM	Systematic mapping
SWL	*Staphylococcus warneri* EX17 lipase
TBU	Enzymatic activity expressed in tributyrin unit
TLL	*Thermomyces lanuginosus* lipase
TLL-IM	Immobilized *Thermomyces lanuginosus* lipase (Lipozyme®TL)
TNBS	Trinitrobenzenesulfonic acid
Tris-HCL	Tris-hydrochloride
TrisAEA	Tris(2-aminoethyl)amine
UNDGLXS	Undecanol-glyoxyl silica
VFR	Vortex flow reactor
VS	Vinyl sulfone

XAD-4	Amberlite resin XAD-4
YLLip2	*Yarrowia lipolytica* LIP2 lipase
ZnP(TLL)-NFs	TLL organic-inorganic zinc phosphate biocatalyst

SYMBOLS

Latin letters

A_{SI}	Peak area of the internal standard	cm^2
C_{KOH}	Concentration of the KOH base	mg mL^{-1}
C_{SI}	Concentration of the internal standard	mg mL^{-1}
d	Reactor diameter	cm
IY	Immobilization yield	%
L	Reactor height	cm
m	Mass of the sample	mg
MW_{KOH}	Molecular mass of the KOH base	g mol^{-1}
RA	Recovered activity	%
R_{in}	Inner radius	cm
R_{out}	Outer radius	cm
t	Reaction time	min
U	Enzymatic activity unit	μmol g^{-1} min^{-1}
V_f	Volume of base consumed on titrating the sample after the reaction	mL
V_i	Volume of base consumed on titrating the sample before the reaction	mL
V_{SI}	Volume of the internal standard solution	mL
w_{enzyme}	Mass of enzyme used in the reaction	g
w_{oil}	Mass oil used in the reaction	g
X_i	Coded independent variables (i = 1, 3)	-
X_j	Coded independent variables (j = 1, 3)	-
y	Predicted response variable	wt.%

Greek letters

β_0	intercept constant coefficients of the model	-
β_i	linear constant coefficients of the model	-
β_{ii}	quadratic constant coefficients of the model	-
β_{ij}	interaction constant coefficients of the model	-
ε	Molar extinction coefficient	M^{-1} cm^{-1}
η	radius ratio	-
ΣA	total peak area of fatty acid ethyl esters	cm^2
Γ	aspect ratio	-

SUMMARY

CHAPTER 1
INTRODUCTION

1.1 Broad context

Current concerns about global warming and the reduction of petroleum reserves have driven the search for chemicals that can be biodegradable and environmentally friendly. Ecofuels (e.g., biodiesel or Ecodiesel) and synthetic biolubricants have become attractive alternatives to petrochemicals [1]. Ecofuels, composed of ethyl or methyl esters of fatty acids, can be produced by transesterification of animal fats or vegetable oils or by direct esterification of fatty acids [2,3] with small-chain alcohols [1,4–6]. While, for example, vegetable oils are promising substitutes to mineral-oil based lubricants after their conversion to fatty acid alkyl esters using long-chain alcohols (C8 to C14), such as octanol [7,8], branched alcohols, such as isoamyl alcohol [9–11] and 2-ethyl- 1 hexanol [12], and polyols [13], such as neopentyl glycol [14], pentaerythritol [15,16] and trimethylolpropane [17,18]. Fatty acid alkyl esters having 22 to 26 carbon atoms can serve as biolubricant components [7] due to their suitable physicochemical properties (some of them after a proper chemical modification of the esters, for example, an epoxidation of C=C double bonds of the unsaturated fatty acid esters [19]), such as high viscosity index, high lubricity, high flash point, low volatility, good anti-wear performance, high thermo-oxidative stability, and good performance at low temperatures [7,11,19,20].

Ecofuels and synthetic biolubricants are obtained by direct esterification of fatty acids [2,3], transesterification between monoalkyl esters of the target fatty acid or glycerides and the desired alcohol [21,22], or hydroesterification, a sequential process of hydrolysis of oils/fats followed by esterification of the purified fatty acids [1,7,14]. These reactions have been performed using alkaline or acid catalysis, although they have some problems, like the production of undesired side-products and the requirement of an initial feedstock with certain features (low acidity, low water content) [23,24]. From an industrial point of view, heterogeneous catalysts are preferred. Immobilized lipases (triacylglycerol acyl-hydrolases, E.C. 3.1.1.3) are good alternative heterogeneous catalysts for the synthesis of these esters due to the low energy consumption, possibility of using unpurified oils (e.g., waste cooking oil containing a high percentage of free fatty acid), and lower complexity of the recovery and purification of the product (lower by-products production) [23,25,26].

There are a great number of techniques and supports suitable for enzyme immobilization. In general, some criteria are followed in their choice regarding to high activity of the immobilized enzyme, high stability against temperature and organic solvents, low cost of immobilization, and low toxicity of the immobilization reagents and supports [27–30].

In the case of lipases, a popular technique that has been widely reported is their immobilization by hydrophobic adsorption on highly hydrophobic surfaces [29,31]. This is because the lipases have a peculiar mechanism, called interfacial activation, a phenomenon that allows the enzyme to exist in two forms in equilibrium: a closed form, in which a lid (polypeptide chain) covers the enzyme active site, and an open form, in which the lid is moved away allowing the lipase to adsorb to hydrophobic surfaces (e.g., drops of oils, air bubbles, etc.) [32–34] and turning the active site accessible to the substrate [34]. This immobilization strategy is simple procedure and allows for one-step immobilization/purification/stabilization and even hyperactivation of lipases. Furthermore, the support can be recovered and reused after enzyme inactivation [29,35]. The set of benefits of immobilization on hydrophobic support make this technique the prime candidate to maintain its supremacy in this area [29].

The main problem with immobilization on a hydrophobic support is the possibility of enzyme desorption when the biocatalyst is subjected to drastic conditions, such as high temperature or the presence of an organic solvent [36]. Furthermore, when applied in biosurfactant synthesis, reactions where biosurfactant is an intermediate product of the process or in heterogeneous medium containing substances with detergent properties [37]. A strategy used to minimize this problem is immobilization on a heterofunctional support. This type of support has a unique surface exhibiting various physicochemical capabilities, a layer of acyl groups to obtain the lipase interfacial activation and a layer of groups able to give other physical interactions (e.g., ionic exchange [38]) or a covalent bond that will make the immobilization irreversible [39–43]. This new biocatalysts are generally even more stable than standard biocatalysts immobilized only by hydrophobic interaction [44].

Another strategy that has been used to minimize problems with hydrophobic supports is the intermolecular crosslinking of immobilized enzymes with physical and/or chemical agents. The association of these agents and the immobilization of the lipase on the support provides the formation of a biocatalyst with the enzyme immobilized by multiple sites [45]. Therefore, for the desorption of the lipase, it will be necessary to release all the aggregates of enzyme/physical or chemical agents. Another advantage of crosslinking with physical or chemical agents is the modulation of catalytic properties and, in some cases, improvements in the stability of the biocatalyst [45].

The preparation of enzyme/metal salts nanoflowers has been established as another strategy that can give heterogeneous lipase biocatalysts and, at the same time, improve enzyme activity and stability in certain cases [46–49]. This strategy is based on the function of some residues of the enzyme surface as crystal nucleation points, generating crystal structures that have the appearance of the petals of a flower, or even of the whole flower (hence the name) after full growth. Although bearing an undeniable interest, the poor mechanical resistance of these nanoflowers is delaying their implementation. To solve this problem, the trapping of the enzyme nanoflower in structures bearing better mechanical resistance has been proposed, together with the use of magnetic materials to facilitate their handling [50–54].

As has been mentioned, there are several techniques for immobilization and stabilization of lipases, however, the appropriate choice of immobilization strategy is conditioned to the peculiarity of the biotechnological process that the biocatalyst will be applied, the reactor to be operated and the technical, economic and environmental impacts that the synthesis of the biocatalyst provides to the process.

1.2 Objectives

The aim of this work was to obtain high performance and stability biocatalysts for application in aqueous and organic reactions. In order to accomplish this aim, the following specific objectives were established:

i) Development of a strategy for the mineralization of immobilized enzymes by interfacial adsorption;
ii) Evaluation of the mineralization process in commercial immobilized enzymes;
iii) Evaluation of the influence of the immobilization technique on the mineralization process of the biocatalyst;
iv) Analysis of adjustments of catalytic properties and stabilization combining physicochemical modification of solid phase and mineralization;
v) Development of a strategy for immobilization on heterofunctional lipase support using resin with octadecyl groups (Purolite$^{@}$ C18);
vi) Application of biocatalysts in biotransformation reactions in aqueous and organic medium.

1.3 Thesis outline

As discussed so far, there are still gaps in existing immobilization techniques and the search for stabilization strategies for immobilized biocatalysts has been evaluated. A review of the literature covering research on the immobilization of lipases on hydrophobic supports was performed using systematic mapping (SM) concepts (Chapter 2). This thesis focuses on the development a library of biocatalysts combining enzyme immobilization and stabilization strategies with mineralization of the immobilized enzymes. Simultaneously, the performance of these biocatalysts in aqueous and organic reactions was also investigated.

In this thesis it was proposed the modification by metal phosphate on already immobilized enzymes as an alternative to solve the nanoflowers fragility while maintaining some of the mineralization benefits. If the enzyme mineralization was performed on previously immobilized enzymes, the researcher can select the support based on its mechanical resistance (and reap the benefits achieved during enzyme immobilization), avoiding the difficulties derived from the management of the small and fragile nanoflowers. Moreover, the mineralization of immobilized enzyme couples the positive effects of enzyme mineralization during nanoflowers production with the benefits of enzyme immobilization in preexisting solids. In Chapter 3, several enzymes were immobilized on octyl agarose beads via interfacial activation and modified with diverse metal phosphates, determining the effects of different treatments on enzyme stability/activity. This study was later extended to the use of diverse commercial biocatalysts (Chapter 4) and TLL immobilized on Purolite C18 (methacrylate beads bearing octadecyl groups) (Chapter 5). The influence of different immobilization and mineralization protocols were performed to verify if the immobilization strategies will permit to analyze if the enzyme structure or orientation regarding the support (or both) may alter the effects of the mineralization (Chapter 6). In Chapter 7, an effort was made to analyze whether the coupled solid phase chemical or physical modification of enzymes and their further mineralization may have additive effects on the enzyme features, the results from the first strategy influencing the results achieved by the second one.

A new research effort has been to prepare a TLL biocatalyst using Purolite$^{@}$ C18, aminating and activating the support surface with vinyl sulfone to achieve covalently immobilized biocatalysts after the first immobilization via interfacial activation, aiming to further improve the enzyme features (Chapter 8). Then apply in transesterification reaction of different vegetable oils with methanol for biodiesel production (Chapter 9). In Chapter 10, Eversa$^{@}$ Transform immobilized on Purolite$^{@}$ C18 was used for the production of fatty acid

alkyl esters. Finally, conclusions, suggestions for future works, and academic production (Chapter 11).

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CHAPTER 2
REVIEWING RESEARCH ON LIPASE IMMOBILIZATION ON HYDROPHOBIC SUPPORTS INCORPORATING SYSTEMATIC MAPPING PRINCIPLES

A review of the literature covering research on the immobilization of lipases on hydrophobic supports was performed using systematic mapping (SM) concepts. This approach consists of a rigorous evidence review methodology used to catalog evidence, identify gaps at the frontier of knowledge, unknown trends, and research groups. Our results showed a wide variety of available lipases, including commercial, wild-type and recombinant strains. However, the most commonly used lipases are lipase B from *Candida antarctica* (CALB), *Thermomyces lanuginosus* (TLL), *Candida rugosa* (CRL), and lipase from *Rhizomucor miehei* (RML). A wide variety of supports with different degrees of hydrophobicity were recovered and the supports activated with octyl and octadecyl groups were the most used. The advantages of immobilization on these supports were discussed. Among them, the immobilization, purification, stabilization and hyperactivation of lipases in a single step. However, problems related to immobilization by interfacial activation were highlighted. Strategies to overcome these problems include immobilization on heterofunctional supports or intermolecular crosslinking of enzymes immobilized by physical and/or chemical agents. The possibility of increasing the capacity of supports by multilayer immobilization was also discussed. Finally, the structure, distribution of the network and the frequency of co-occurrence between lipases and supports were elucidated to determine the possible hotspots and frontiers of knowledge not yet explored.

2.1 Introduction

Biocatalytic processes have been expanding due to their promising role in the development and optimization of industrial technologies for biotransformation reactions. Among the most used biocatalysts, lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are gaining a privileged position due to their numerous advantages over the use of traditional chemical catalysts, especially those concerning the high specificity of these enzymes [1–5]. However, the use of such enzymes in their soluble form for large-scale industrial processes is not very attractive, because of their high production cost and low operational stability [6]. To overcome these drawbacks, the immobilization of these biocatalysts on a solid support has been widely exploited. If properly designed, this approach can provide several advantages from an

industrial point of view, such as an increase in the biocatalyst operational stability, easy recovery from the product stream, and the possibility of lipase reuse [6–10].

These enzymes have been extensively immobilized by adsorption on highly hydrophobic surfaces [11–15] due to their peculiar mechanism, which is called interfacial activation (Figure 2.1). It consists in the existence of the lipase in two forms in equilibrium: 1) a closed form (hydrophobic regions of the lid interact with hydrophobic regions around the active center, isolating it from the reaction medium); 2) an open form (the lid moves and allows the lipase adsorption on hydrophobic surfaces (e.g., drops of oils, air bubbles, etc.) [16–19], exposing the active center to the medium) [19].

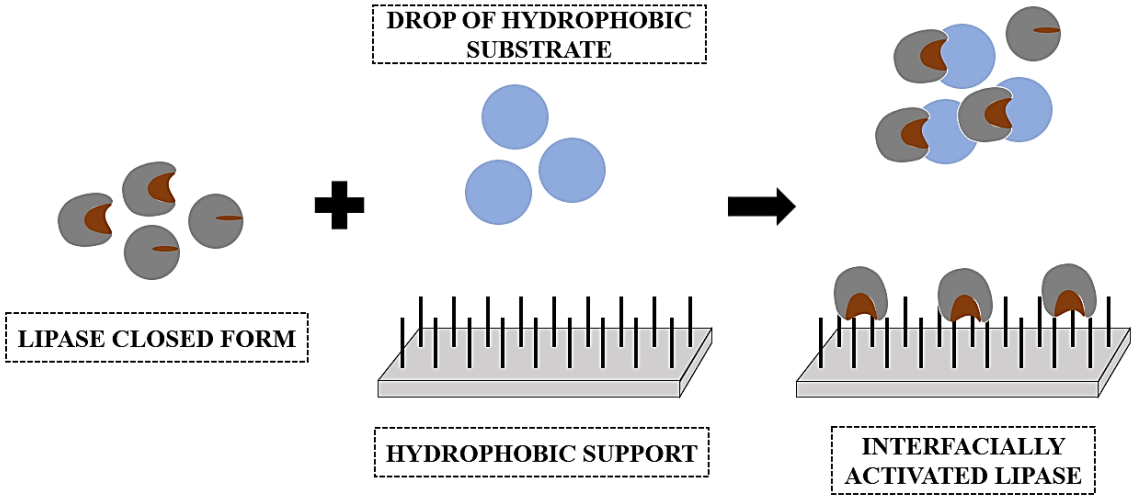


Figure 2.1. Interfacial activation of lipases versus drops of insoluble substrate and hydrophobic support.

This immobilization protocol is a simple technique and presents several advantages: 1) pH is not a limiting factor as long as the enzyme is soluble and stable at the working pH [20]; 2) low ionic strength can lead to the simultaneous immobilization and purification of lipases [20–24]; 3) the immobilization method is reversible; 4) the open and adsorbed form of this enzyme is very stable [25–27], even regarding covalently immobilized multipoint lipases [28,29]; 5) lipases immobilized on hydrophobic supports have the active center more exposed to the medium and the lid does not move in the presence of high ionic strength medium [30]; 6) reduction in the accumulation of hydrophilic compounds on the biocatalyst surface [31–33]; 7) Higher enzymatic activities can be achieved regarding the free enzyme, due to the lipase hyperactivation during the immobilization step [21].

Moreover, the relevance of the research addressing the immobilization of lipases on hydrophobic supports is indisputable, since this theme has led to an increased number of published scientific research papers over the years (Figure 2.2) [20,34]. Accordingly, in an effort to advance with rigorous methods for conducting evidence reviews in this topic, we incorporated some principles of systematic mappings to evaluate the research involving the immobilization of lipases on hydrophobic supports. SMs methodologies investigate and reveal trends, knowledge gaps, and existing heterogeneity in studies conducted on benches. This is because SMs retrieve comprehensive research from various bibliographic sources, transparently screens articles, and critically assesses the quality of these studies [35–39].

Guidelines applied to standardize SM methods are formulated by formal coordinating review bodies from various disciplines, such as Cochrane in healthcare, the Campbell Collaboration in social welfare, and the Collaboration for Environmental Evidence (CEE) in conservation and environmental management [40]. Nonetheless, there is no specialized organization devoted to guiding the conduct of evidence synthesis in the chemical engineering sector, which suggests that the majority of reviews in this area are classified as nonsystematic. Consequently, comprising SMs methods to conduct a literature review in this field may allow the extraction of relevant information from our research subject.

In this context, the purpose of this review is to present trends and gaps across the literature on the immobilization of lipases on hydrophobic supports in order to subsidize future studies on this topic. Our evaluation is restricted to a specific subset of scientific articles retrieved from four bibliographic sources: Web of Science (Core Collection: SCI-E and ESCI), Scopus, PubMed (Central; PMC), and SciELO.

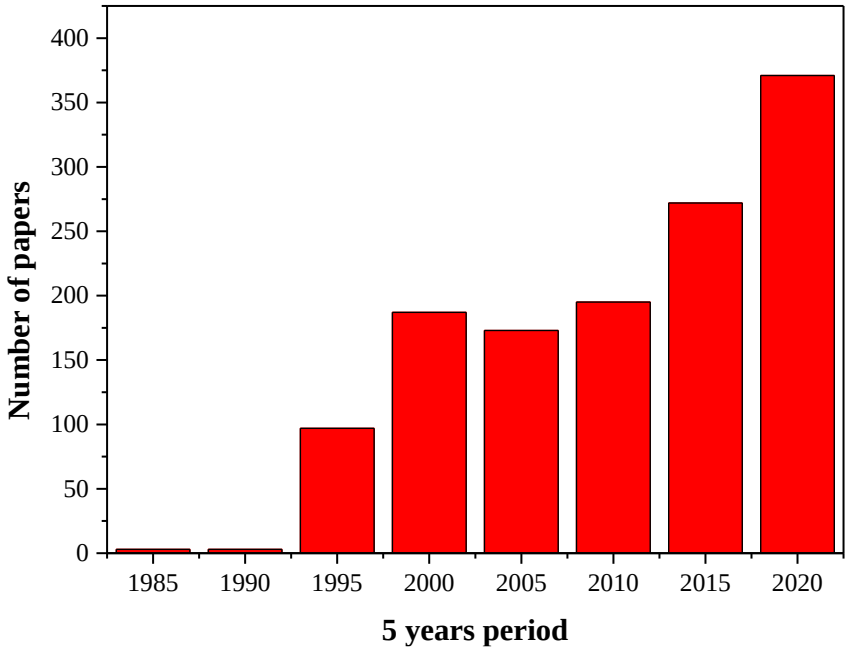


Figure 2.2. Annual distribution of all records retrieved through the bibliographic searching about the immobilization of lipases on hydrophobic supports in the Web of Science.

2.2 Methodology

2.2.1 Literature searching and database building

The design of this review was performed by following the Collaboration for Environmental Evidence guidelines (Collaboration for Environmental Evidence, 2018), in close collaboration with environmentalists and specialists on the subject. The following search string was used to retrieve titles, abstracts, and keywords of related publications: ("interfacial activation*" OR "activation immobilization*" OR "interfacial immobilization*" OR "interfacial activation immobilization*" OR "interfacial adsorption*" OR "adsorption immobilization*" OR "hydrophobic adsorption*" OR "hydrophobic immobilization*" OR "hydrophobic adsorption immobilization*" OR "hydrophobic support*" OR "hydrophobic interaction*") AND "lipase*". Searches were restricted to articles published between 2011 and 2020 in order to obtain a sample of recent publications. The database was updated on April 26th, 2021 (see the complete searching procedures in Figure A2.1 – Supplementary Material).

Bibliographic searches were performed in four different databases to minimize possible biases into the review process, returning a total of 1855 articles. We retrieved 368 publications from Scopus, 644 from Web of Science, 843 from PubMed, and none from SciELO (Table A2.1 – Supplementary Material). After an analysis of coverage and overlapping to remove

replicates, the database was reduced to 1433 publications. Eligibility criteria were used to eliminate unrelated publications inadvertently included in the reference list after this search. Initially, the screening process was conducted analyzing titles and abstracts, considering the following inclusion criteria: (i) lipase immobilization; (ii) lipase immobilization on solid supports; (iii) lipase immobilization on hydrophobic supports. A total of 370 records were then submitted to a full-text analysis and 264 primary studies were selected to be included in this study. A summary of the employed methodology is presented in Figure 2.3 and details of the papers eliminated after title/abstract screening and full-text analysis are shown in Table A2.2 and Figure A2.2 (Supplementary material), respectively.

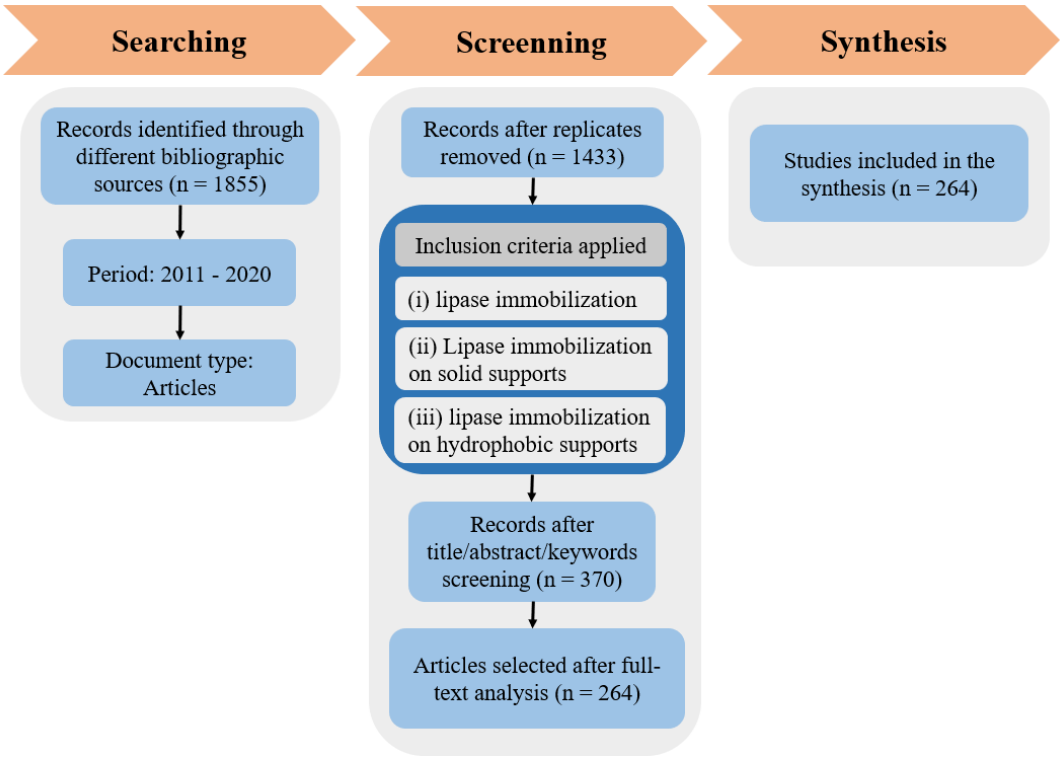


Figure 2.3. Flow Diagram for the selection of studies (Adapted from ROSES Flow Diagram for Systematic Maps. Version 1.0).

A sample of 30 random articles was double screened for two different assessors (J.R.G. and M.C.P.G.) to account for subjective decisions in the inclusion/exclusion of eligible studies (Collaboration for Environmental Evidence, 2018). Decisions were compared using the Kappa test of agreement test to ensure the repeatability of the process [41] and a Kappa score of 92% (95% Lower/Upper Confidence Limit) was obtained, which indicates almost perfect agreement

between the reviewers, as well as decisions sufficiently repeatable (Table A2.3 - Supplementary Material).

2.2.2 Data coding and data synthesis

Articles meeting our inclusion criteria (n = 264) were subjected to data coding and synthesis of results. Two categories of keywords (types of lipase and hydrophobic support) were created and assigned to each paper to build the network map. Despite the extensive literature that exists on immobilization of lipases on hydrophobic supports, important aspects of this immobilization protocol remain to be clarified. Considering this, here we elucidated some of the relevant aspects, including the main enzymes and supports used, besides problems and solutions that are related to this research topic.

2.2.3 Data handling

For data processing, Origin® (version 9.0) and MS Excel (v. 2016) were used to perform the calculations and prepare the graphs. The software R was used to perform the screening process - 'revtools package' (Core, 2019). Furthermore, the set of keywords created were applied to the VOSviewer software (version 1.16.15) through the criterion of co-occurrence of terms to build network maps containing information related to the important topics of immobilization of lipases on hydrophobic supports.

2.3 Results and Discussion

2.3.1 Enzymes and supports used in the hydrophobic immobilization

The immobilization of lipase on a hydrophobic support can allow the synthesis of a library of biocatalysts with modulated catalytic properties for application in industrial biotransformation processes. This is possible due to the structure and properties of the different existing supports [42–45] and the affinity of the lipases with these carriers [46,47], as well as the working conditions involved in the immobilization [48,49]. These factors, alone or collectively, can alter the selectivity, specificity and final activity of lipases and in some cases lead to immobilized biocatalyst design much more suitable for the production process than the free enzyme formulation [49–53]. Thus, there is no way to say whether a specific lipase is adequate or inadequate for a specific process [52].

As shown in Figure 2.4, the most used enzymes in immobilization on hydrophobic support are Lipases B from *Candida antarctica* (CALB), *Thermomyces lanuginosus* (TLL) and

Candida rugosa (CRL). Probably, these lipases have been used due to their stability after immobilization. CALB and TLL are enzymes that have high stability and can be used in various industrial conditions, and are already commercialized as immobilized formulations in resins [54,55]. On the other hand, CRL presents moderate stability [56] and an immobilized formulation is not available on the market. The use of stability is a delicate factor to infer the sovereignty of these lipases in this bibliographic survey, considering that lipase A from *Candida antarctica* (CALA) has high stability and is in the seventh position of the most used lipases in the survey. In addition, Eversa@ Transform 2.0 (EV2.0) is an evolution of TLL and has high stability, but is not in the Top 10 due to its recent appearance in the market [57].

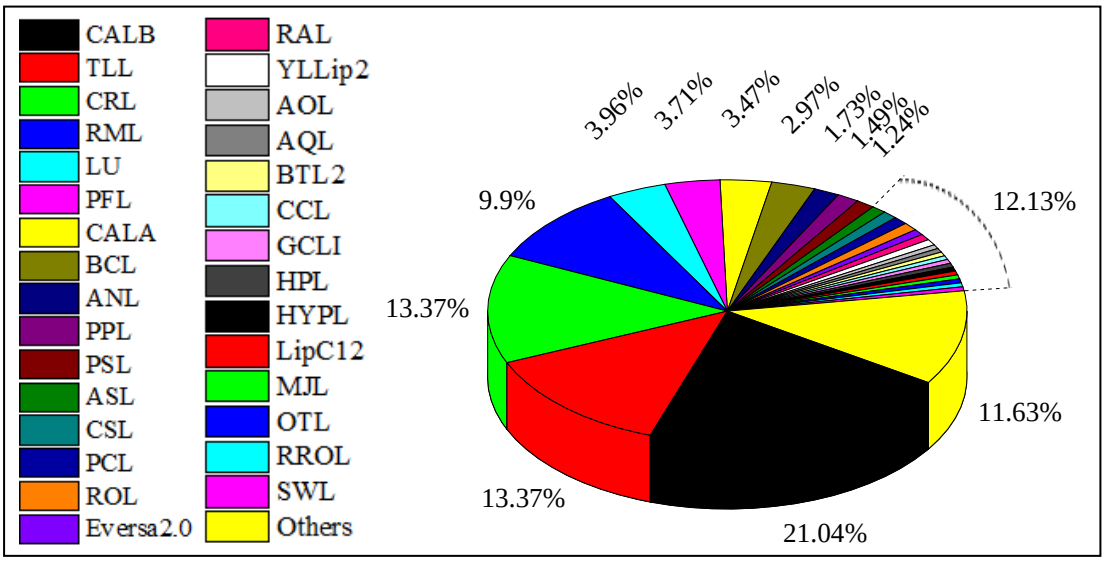


Figure 2.4. Lipases used for immobilization by interfacial adsorption. The category others represents lipases or hydrophobic supports with less than 1% of occurrences in our dataset.

The cost of acquiring enzymes is a factor that reduces the technical and economic viability of biocatalytic processes, it is probably not the main variable that determined the ranking of lipases. Lipases such as CRL and porcine pancreas lipase (PPL) are formulations that have a low cost compared to other lipases [56,58], but PPL is the tenth most used lipase in the bibliographic survey. Probably, the specificity of these lipases is the main factor that led to the classification of this survey. Non-specific enzymes can be applied in numerous industrial processes, however, enzymes that present regiospecificity are limited to restricted reactions in industry, but no less important than the others. Some lipases, such as TLL and Eversa@ Transform, are 1,3-specific enzymes whose specificity is affected when immobilized mainly on hydrophobic supports. Thus, this increases the range of application of these enzymes [14,49].

Figure 2.5 shows a compilation of the most used hydrophobic supports from 2011 to 2020. It is possible to observe the wide variety of matrices with different hydrophobic degree used for lipase immobilization. Among them, there are commercial supports, such as octyl-agarose, EC-octadecyl Sepabeads™, Accurel MP 1000, and even Lewatit VP OC 1600 used in the synthesis of Novozym® 435 [54]; functionalized supports based on pre-existing support, such as octyl silica, octyl-glyoxyl agarose, silica modified with phenyl; however, the use of supports prepared from agro-industrial residues is also observed, such as rice husk silica (RHS) with triethoxy(phenyl)silane, chitin-polyhedral oligomeric silsesquioxanes support, cellulose/Fe_2O_3 hydrogel microspheres.

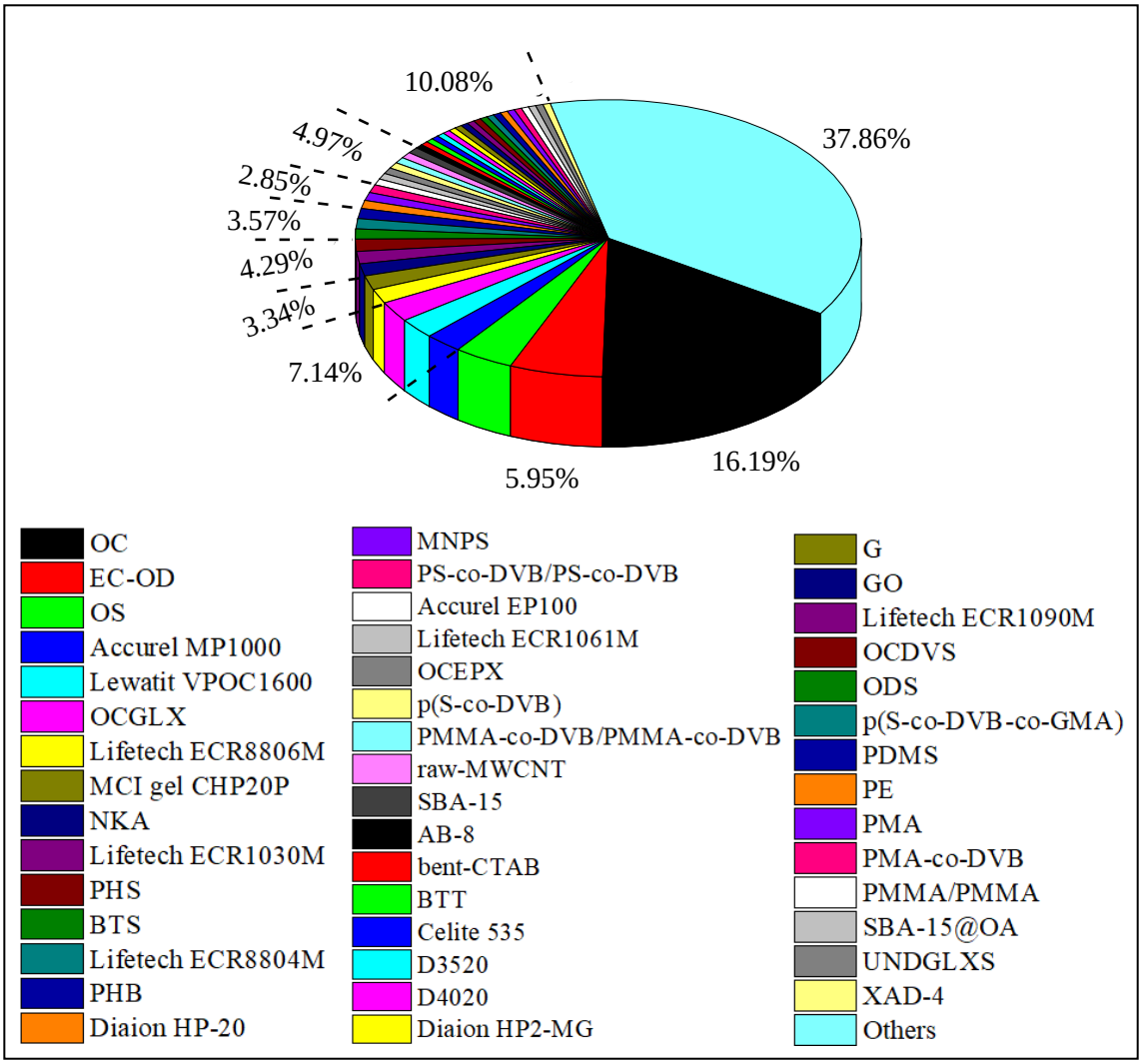


Figure 2.5. Hydrophobic supports used for immobilization by interfacial adsorption. The category others represents lipases or hydrophobic supports with less than 1% of occurrences in our dataset.

Given the variety of available supports, a library of biocatalysts with different properties for industrial application can be obtained. This is only possible because the structure and properties of the supports condition the catalytic properties of the lipases. This effect can produce changes in the specificity and selectivity of the enzymes, modulating the biocatalyst to the desired biotechnological process [59–61].

The main acyl groups present on supports are the octyl and octadecyl groups. In general, the use of these acyl groups can lead to the formation of a biocatalyst with different properties, even using the same conditions during hydrophobic immobilization. TLL was immobilized on octyl-agarose (OC) and Purolite@ C18 (Lifetech ECR8806M) and the results demonstrated that the biocatalyst immobilized on resin with octadecyl groups led to better modulation in the catalytic properties and stabilization when compared to the biocatalyst immobilized on OC [62]. Furthermore, the greater degree of hydrophobicity of octadecyl groups can improve purification and simultaneous immobilization with greater recovery of catalytic activity [23,63].

2.3.2 Problems of lipase immobilization on hydrophobic support

The main problem with immobilization on a hydrophobic support is the possibility of enzyme desorption when the biocatalyst is subjected to drastic conditions, such as high temperature or the presence of an organic solvent [64]. Furthermore, when applied in biosurfactant synthesis, reactions where biosurfactant is an intermediate product of the process or in heterogeneous medium containing substances with detergent properties [65]. A strategy used to minimize this problem is immobilization on a heterofunctional support. This type of support has a unique surface exhibiting various physicochemical capabilities, a layer of acyl groups to obtain the lipase interfacial activation and a layer of groups able to give other physical interactions (e.g., ionic exchange [66]) or a covalent bond that will make the immobilization irreversible [67–71]. This new biocatalysts are generally even more stable than standard biocatalysts immobilized only by hydrophobic interaction [72].

The use of heterofunctional support can provide different modulating effects on the catalytic properties of lipases due to changing the orientation of the enzyme during immobilization [73], providing co-immobilization of enzymes [74], and increasing the possibilities of application of the biocatalyst in industry [72,75,76]. The limitation of the use of these supports is related to the sensitivity of the enzyme to alkaline pH when subjected to covalent immobilization [67]. Usually on heterofunctional supports, initially the enzyme is immobilized by interfacial activation under conditions of low ionic strength and pH in the range of 5.0-7.0, and later, the conditions are modified to favor covalent bonds, such as incubation in

alkaline solution. Some supports and their functional groups are presented in this review, as shown in Table 2.1.

Table 2.1. Functional groups present in heterofunctional supports used in the immobilization of lipases by interfacial activation and covalent bonding.

Lipase source	Support	Functional groups	Reference
CRL; RML; TLL	OCDVS	Octyl and vinyl sulfone	[76]
CALB	LifetechTM ECR 8285F	Butyl and epoxy	[77]
CALB	SMMP-octyl-glu	Octyl and aldehyde	[78]
CRL; LipC12	Aga-C8-GLU	Octyl and aldehyde	[79]
BaL	GO-NH2- PMAO	Hydrophobic groups present on the support and aldehyde	[80]
PLL	PBA−PAD	Inner hydrophobic PBA domain and aldehyde	[81]
CRL	Lifetech™ ECR1030M; Lifetech™ ECR8285	Butyl and epoxy	[82]
CRL; CALB; RAL; ASL; HPL; MML; PCL; RNL	P(SAN-DVB)-GMA	Hydrophobic groups of the matrix poly(styrene-co-acrylonitrile-co-divinylbenzene) and epoxide	[83]
LipC12	OCA; ODA	Octyl and aldehyde; Octadecyl and aldehyde	[84]
SHL	PMA-co-DVB	Inner hydrophobic PBA domain and aldehyde	[85]
CALB; CCL; PCL; PFL; HPL	ChiS-G	Hydrophobic portion of chitosan and aldehyde	[86]
RML	OCEPX	Octyl and epoxide	[87]
CALB	UndGLX, OCEPX; UndGLXS; OCEPXS	Undecanol and glyoxyl; octyl and epoxide	[70]
BTL2	UndGLXS; OCEPX	Undecanol and glyoxyl; Octyl and epoxide	[88]
PsL; AsL	OGS	Octyl and glyoxyl	[69]
CALA; CALB; CRL; TLL; RML; LU	OCDVS; OCGLX	Octyl and vinyl sulfone; octyl and glyoxyl	[72]
CALB	OCGLX	Octyl and glyoxyl	[75]
CALB; TLL	OCGLX	Octyl and glyoxyl	[89]
LU; RML; PFL	OCGLX	Octyl and glyoxyl	[74]
CALB; TLL; RML	OCGLX	Octyl and glyoxyl	[90]
PFL	OCGLX	Octyl and glyoxyl	[91]

CRL; CALA	OCGLX	Octyl and glyoxyl	[68]
RML; CRL	OCGLX	Octyl and glyoxyl	[92]
CALB; TLL; RML	OCGLX	Octyl and glyoxyl	[67]
CALB; RML; LEU	OCGLX; OCGLXR	Octyl and glyoxyl	[93]
PsL; AsL	OCGLXS	Octyl and glyoxyl	[94]
CALA; CALB; TLL; RML; CRL; LU	OCEDA; OCHDA	Octyl and glyoxyl	[66]

Table 2.1 demonstrates the predominance of supports activated with octyl and glyoxyl groups for the immobilization of different lipases, followed by the use of supports activated with octyl and epoxy groups. The other heterofunctional supports present distinct groups for immobilization by interfacial and covalent activation. The support activated with octyl and vinyl sulfone groups has not been used frequently, however, it should be highlighted as a support that can lead to the construction of a library of biocatalysts. This is possible because at the end of the covalent immobilization, there is a need for a final step that requires the blocking of the other reactive groups of the support with a nucleophile. This step allows the adaptation of enzyme-support interactions using reagents with very different physical properties. Thus, the use of different blocking reagents can lead to the formation of biocatalysts with different structures and functional properties, even starting from a collection of immobilized enzymes with exactly the same distribution of enzymes present on the support, orientation in relation to the support surface and number of binding of enzymes to the support [29,95,96], shown in Figure 2.6.

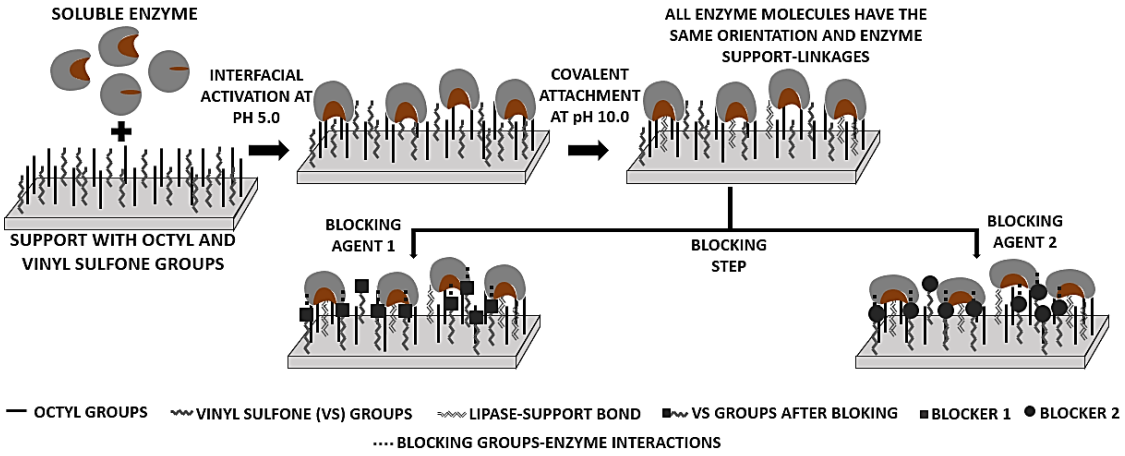


Figure 2.6. Schematic representation of enzyme immobilization on octyl and divinyl sulfone groups heterofunctionalized support.

The most used acyl group in the supports is predominantly octyl, however, the octadecyl group can lead to better lipase stabilization [62]. No paper showing the combination of octadecyl and vinyl sulfone groups in a heterofunctional support was retrieved in the systemic analysis, as well as no record was found when performing a brief analysis of data from the last years extracted from the Web of Science using "octadecyl" AND "vinyl sulfone" as a search criterion. This is a gap on the frontier of knowledge to be explored for the study of the synthesis of heterofunctional supports with these characteristics. A possible problem is the size mismatch of the spacer spleens of vinyl sulfone and octadecyl groups. In this case, strategies must be sought to minimize this problem.

Another strategy that has been used to minimize problems with hydrophobic supports is the intermolecular crosslinking of immobilized enzymes with physical and/or chemical agents. The association of these agents and the immobilization of the lipase on the support provides the formation of a biocatalyst with the enzyme immobilized by multiple sites. Therefore, for the desorption of the lipase, it will be necessary to release all the aggregates of enzyme/physical or chemical agents. Another advantage of crosslinking with physical or chemical agents is the modulation of catalytic properties and, in some cases, improvements in the stability of the biocatalyst. However, recovery of the support after enzyme inactivation becomes complicated if intermolecular cross-linking of all enzyme molecules is achieved. Table 2.2 shows the main physical and/or chemical agents used in the papers obtained in the bibliographic survey.

Table 2.2. Building-block chemicals used for the modification of the enzymatic surface after immobilization by interfacial adsorption.

Lipase source	Support	Chemical or physical agent	Reference
TLL	Lifetech™ ECR8806M; EC-OD; Lewatit VP OC 1600	PEG	[14]
CALB; TLL; RML	OCGLX	EDA	[90]
TLL	MNPs	EDA, GA and DA	[97]
CALA; CALB; RML; TLL; LU	OC	PEI and GA	[98]
RML	OC	DA	[64]
CALB; RML	OC	PEI and DS	[99]
CALB	OC	PEI	[100]
CALB	OC	DA	[101]
AFE	EC-OD	GA	[102]
RML	OC	poly-allylamine (PAA) and DA	[103]
CALB	OC	EDA and TNBS	[59]

CALB; TLL; RML	OC	EDA, amino groups with succinic anhydride and PEI	[61]
CALA; CALB; TLL; RML; LU	OC; OVS	PEI	[104]
LipC12	OCA; ODA	PEG	[84]
CALB	Lewatit VP OC1600	TNBS, EDA, PEI	[105]
CALB	OC	PEI and DS	[106]
CALB	NKA	PEG	[15]
RML	OC	PEI and DS	[107]
CALB; TLL; RML	EC-OD	PEI and DS	[60]
CALB	OC	PEI	[108]
TLL; RML; CALB	OC	EDA and DA	[109]
CALB; TLL; RML	Lewatit VP OC1600	PEI	[110]
TLL	EC-OD	PEG	[111]
LU	OC	EDA and TNBS	[112]
CALB	OS	PEG	[113]
CALA; CALB; RML; TLL; LU	OC	PEI and GA	[114]
CALB	PTMOS	GA	[71]
CRL	PHB	GA	[115]
CRL	MSU-H	GA	[116]
Eversa2.0; CALB	OC	PEI	[117]
CALA; CALB	OC	PEI	[118]

Physical or chemical agents have different characteristics. Glutaraldehyde (GA) is a small molecule that has been used in situations where the enzymes are immobilized at high load on the support [71,102,115,116] or when the surface of the immobilized enzyme is aminated with ethylenediamine (EDA) [112], since close proximity between molecules is required or that the groups amines are located too close together for intermolecular crosslinking to be efficient [119]. On the other hand, dextran aldehyde (DA) is a large molecule that does not demonstrate the limitations involved with crosslinking with glutaraldehyde, however, due to being a multifunctional reagent, it provides a very strong crosslinking that makes immobilization of the enzyme on the support irreversible. This makes it impossible to reuse the support after enzyme inactivation [34]. Poly-ionic polymers, such as polyethyleneimine (PEI) or dextran sulfate (DS), are alternatives to dextran aldehydes, as they provide highly efficient crosslinking, but do not preclude the reuse of the support after enzyme inactivation [99,106,107].

The cross-linking of the enzyme immobilized on a hydrophobic support has been carried out with physical-chemical agents of carbonic origin, however, a possibility to be studied is the cross-linking of immobilized lipases with metal phosphate. This strategy arises from the frontier of knowledge related to the use of metallic phosphate for free enzyme mineralization by the immobilization technique called nanoflower. In this case, the metal phosphate interacts with a protein nucleation center and starts the self-assembly of an organic-inorganic flower leading to the formation of an insoluble biocatalyst. Expanding this approach to the immobilized enzyme, an immobilized biocatalyst with intermolecular crosslinking using inorganic agent can be obtained. In this case, the metal phosphate binds to the enzyme nucleation point and begins the growth of the inorganic structure with formation of an inorganic film on the immobilized enzyme. Probably, the nanoflower self-assembly step is not reached because the enzyme is immobilized in a plane. The combination of immobilization and inorganic crosslinking can unite the benefits of both protocols and contribute to the formation of a biocatalyst with catalytic properties and greater stability than the biocatalyst without crosslinking.

Another problem that is related to all supports, not restricted to hydrophobic supports, is their load capacity, that is, the amount of enzyme that can be immobilized per gram of support. If the carrying capacity of the support is increased, its impact on the total cost of biocatalysts can be reduced [120]. An alternative is the construction of biocatalysts with a three-dimensional design composed of multilayers of enzymes (an enzyme layer over the previous one to multiply the final load capacity of the support) after enzyme immobilization. This allows the use of a smaller amount of support by increasing the volumetric or mass activity of the biocatalyst [104,114]. In addition, this strategy allows the synthesis of combilipases that can be used in a cascade reaction.

PEI is a crosslinking agent that can be used to build a multilayer biocatalyst starting from an immobilized enzyme. This polymer is able to adsorb onto immobilized enzymatic molecules through strong ionic exchange. In addition, the PEI-coated enzyme is able to immobilize other enzyme molecules also via ion exchange [98,100,104]. Thus, enzymes immobilized on PEI become three-dimensional beds [121]. After each layer overlapping, crosslinking with glutaraldehyde of the structural network formed by PEI and enzyme can be carried out; this strategy of immobilization by covalent bonding is adopted to prevent the release of the enzyme when subjected to drastic operating conditions [98]. Dextran sulfate is also an alternative polymer to PEI in the construction of multilayer biocatalysts, but it has not been evidenced in the literature.

2.3.3 Co-occurrence analysis of keywords

We performed a co-occurrence analysis between the main lipases and supports in our database. Based on the retrieved publications, it is possible to elucidate the structure, distribution of the network and the frequency of co-occurrence of these keywords to determine the possible hotspots and frontiers of knowledge not yet explored.

Bibliometric maps revealed that 75 lipases and 45 hydrophobic supports were cited by the studies we investigated (Figure 2.7). Figure 2.7a shows the use of more than one lipase in ~23% of the studies, which may indicate that these studies are looking for an enzyme with catalytic properties suitable for the productive process being evaluated, since there is no universal biocatalyst for application in several processes. CALB showed the highest number of co-occurrences with different lipases (N = 34), the main ones being TLL, CRL, RML, PFL, CALA and Lecitase Ultra (LU). Next, RML (N = 32), TLL (N = 28) and CRL (N = 17) were the lipases that showed the highest co-occurrence. The use of different lipases in the same publication may be related to the development of new methods of immobilization and/or stabilization of the final biocatalyst, making it necessary to validate these strategies with different lipases that have different characteristics. In addition, it can demonstrate the need to develop a library of biocatalysts with suitable catalytic properties for the industrial process to be studied.

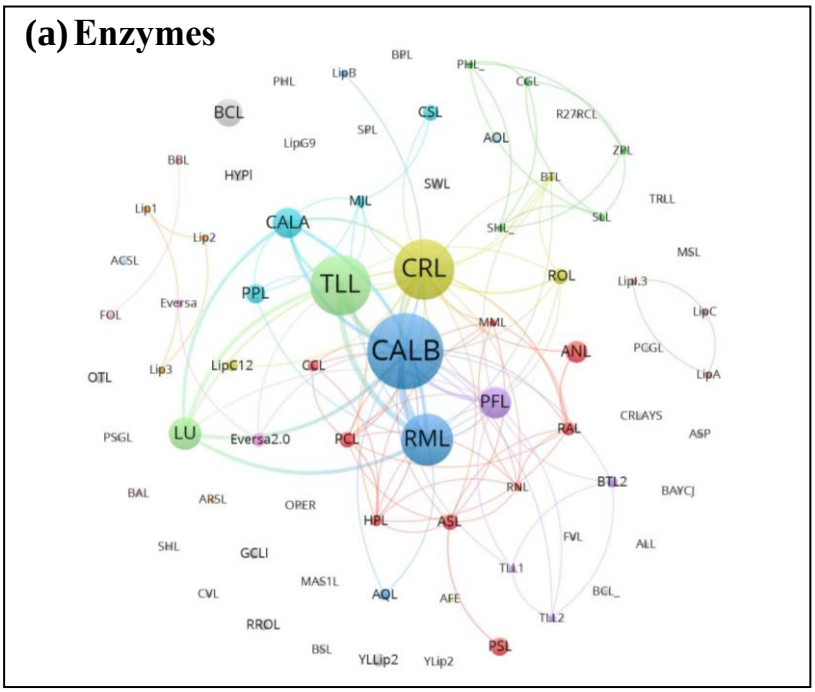
As can be seen in this systemic analysis, several studies are focused on the immobilization of CALB, TLL, CRL, RML, PFL and CALA for application in industrial processes or studies of new stabilization strategies for these enzymes. However, Eversa Transform is a new lipase on the market that has been gaining space in several applications in aqueous and organic media and only presented 3 co-occurrences. This is an enzyme that emerged as an improved version of TLL and has a low cost compared to the mentioned enzymes, high thermal stability and greater resistance to the presence of methanol [57]. Another enzyme worth mentioning is PPL, which presented only one co-occurrence. This enzyme has a very low cost, so this factor associated with the immobilization technique by interfacial adsorption can minimize the costs of the process and carry out the simultaneous purification and immobilization of this enzyme. The main limitation is its specificity, which is lipase 1,3-specific [58].

In Figure 2.7b, a co-occurrence network between hydrophobic supports occurs in ~30% of publications. This result suggests that the use of supports with different degrees of hydrophobicity served as priorities in studies to obtain biocatalysts with different catalytic properties. We found a high number of co-occurrences between commercial and non-

commercial supports (N = 22). This demonstrates two possible issues, the first would be the use of commercial support to validate the importance of non-commercial support; the second would be to use commercially pre-existing supports, aiming at ease of acquisition and, possibly, reduction of process costs. There is also the co-occurrence of heterofunctional supports (N = 5) and the occurrence of many supports prepared using materials that are agro-industrial residues, although there is no co-occurrence between them. Even though supports prepared from agro-industrial residues are evident, there is a gap and the need to explore other materials, such as glycerol, lignin, soybean husk, among others. These residues can be used for the synthesis of activated carbon, which are rich in oxygen and nitrogen surface groups, and can be used *in natura* or activated with desired reactive groups [122,123].

2.3.4 Limitations

Our strategy was mapped using two Web of Science databases (SCI-E and ESCI), Scopus, PubMed and SciELO. We chose not to use scientific evidence, such as theses and dissertations, committee or government reports, documents from congresses, conferences and seminars, and ongoing research. This limits it to a set of primary data published in scientific journals, that is, the information extracted from publications obtained in the bibliographic survey may have been underestimated. Thus, our conclusions must be evaluated under these circumstances. However, the contribution of this review is very important to show the direction of research related to the immobilization of lipases on hydrophobic supports.



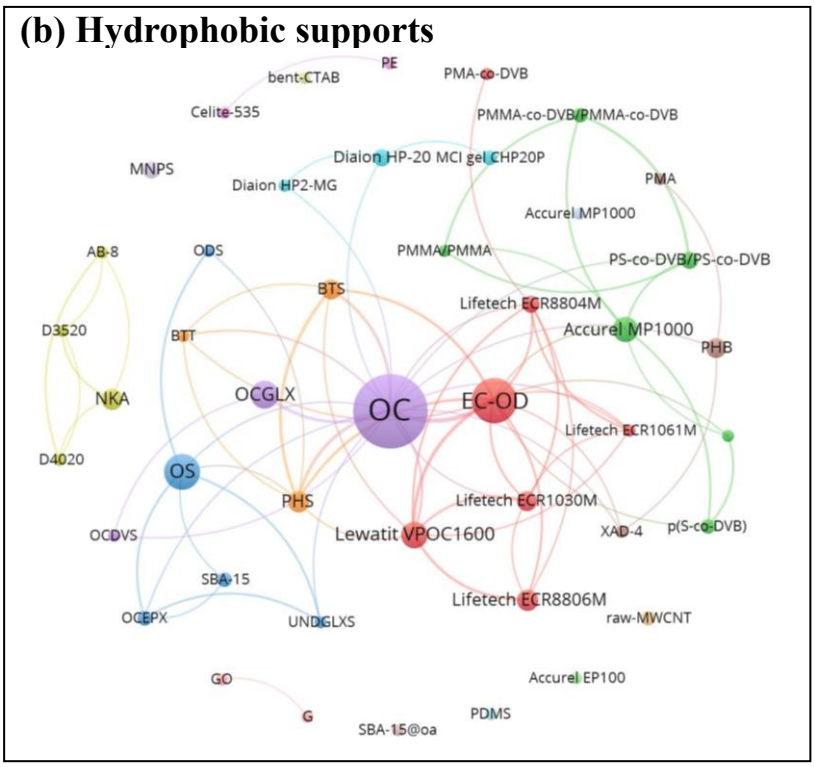


Figure 2.7. Network analysis of lipases (a) and hydrophobic supports (b) addressed in our dataset for the enzymatic immobilization by interfacial adsorption. The size of the node is proportional to the number of occurrences, and the thickness of the edges represents co-occurrences between items. The map of hydrophobic supports was built with at least two occurrences.

2.4 Conclusions

The results presented in this research demonstrate a systematic review of the immobilization of lipases on hydrophobic supports. Here, a wide variety of lipases with different applicability are presented. It is not possible to obtain a universal biocatalyst for use in different applications, however, the choice of support and the enzyme immobilization and stabilization strategy are factors that can influence the catalytic properties and final stability of the immobilized biocatalyst. Various supports with different degrees of hydrophobicity are presented. Furthermore, the problems related to immobilization by interfacial adsorption and the strategies used to overcome them are described. Immobilization on a heterofunctional support and crosslinking with a physical or chemical agent are the main solutions to this problem. A co-occurrence analysis between the main lipases and supports in our database was performed. Furthermore, the structure, network distribution and frequency of co-occurrence

between lipases and supports to determine possible hotspots and yet unexplored frontiers of knowledge were elucidated. Therefore, the contribution of this review is very important to show the direction of research related to the immobilization of lipases on hydrophobic supports, their bottlenecks and solutions to problems.

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APPENDIX

Table A2.1. Search strategy for locating articles. All searches were undertaken on April 26th, 2021.

Bibliographic sources	**Search String**
Searched terms (2011-2020, Articles)	("interfacial activation*" OR "activation immobilization*" OR "interfacial Immobilization*" OR "interfacial activation immobilization*" OR "interfacial adsorption*" OR "adsorption immobilization*" OR "hydrophobic adsorption*" OR "hydrophobic immobilization*" OR "hydrophobic adsorption immobilization*" OR "hydrophobic support*" OR "Hydrophobic interaction*") AND "lipase*"
Web of Science	Basic search: TOPIC (Core collection: SCI-E, ESCI) **644 records**
Scopus	Basic search: TITLE-ABSTRACT-KEYWORDS **368 records**
PubMed	Basic search: ALL FIELDS **843 records**
SciELO	Basic search: TOPIC (Citation Index) **No records**
Total records	1855

Table A2.2. Records eliminated after title/abstract screening.

Categories	Number of publications
Reviews	182
Application of free enzymes in bioprocesses	48
Lipase production without immobilization or immobilized in the whole cell	28
Immobilization of other enzymes (α-amylase, protease, laccase, among others)	58
Use of lipase for medical processes	36
General studies with free enzymes	329
Use of commercial immobilized enzymes	14
Surface modification of the free lipase	10
Others*	357
Total records	1062

*In the category others: use of constituents as a lipase inhibitor; poly(R)-3-hydroxybutyrate (PHB) studies; vaccine production; general studies on proteins; studies with liposomes; immobilization of other compounds; molecular dynamics simulations with lipase; protease production; use of lipase to agents with functional properties for humans; general studies with microorganisms; synthesis of compounds in general; studies related to diseases in general.

Table A2.3. Interpretation of Kappa statistic.

Kappa	Interpretation
<0.0	No agreement
0.0 - 0.20	Slight agreement
0.21 – 0.40	Fair agreement
0.41 – 0.60	Moderate agreement
0.61 – 0.80	Substantial agreement
0.81 – 1.00	Almost perfect agreement

Fonte. Okwuashi *et al*., 2012.

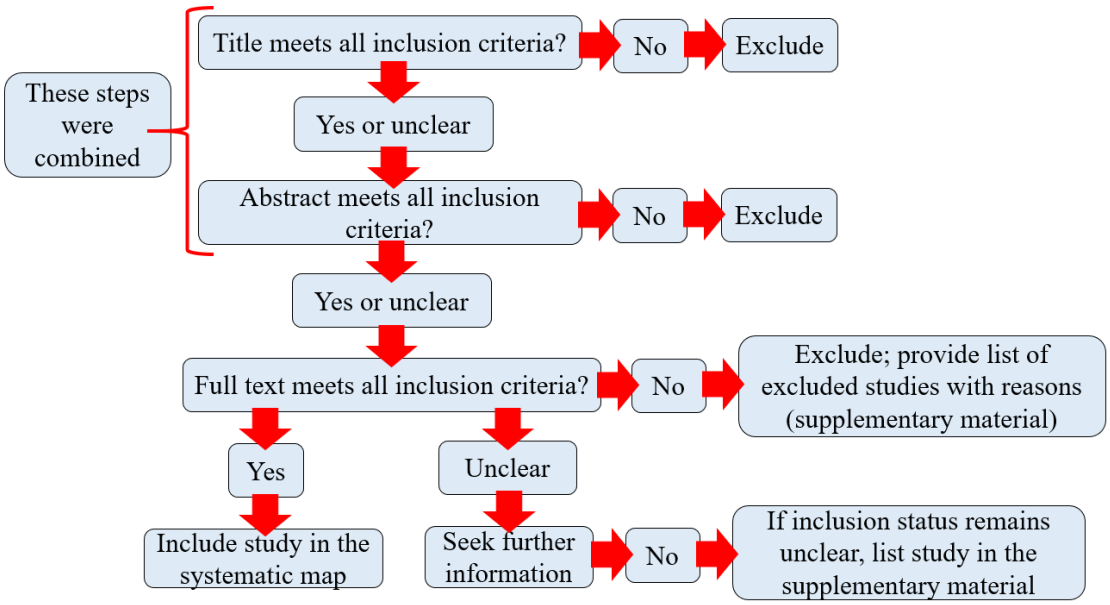

Figure A2.1. Stages of the literature screening process (Adapted from CEE, 2020; Available at: http://www.environmentalevidence.org/guidelines/section-6).

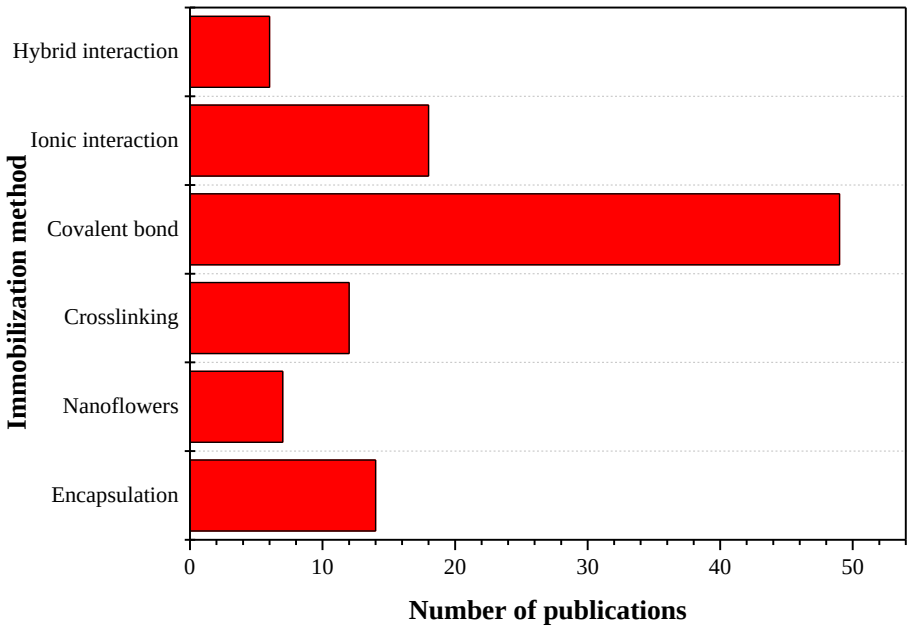

Figure A2.2. Records eliminated after full-text analysis.

CHAPTER 3
STABILIZATION OF IMMOBILIZED LIPASES BY TREATMENT WITH METALLIC PHOSPHATE SALTS

Lipases from *Thermomyces lanuginosus* (TLL), *Rhizomucor miehei* (RML), *Candida rugosa* (CRL), forms A and B of lipase from *Candida antarctica* (CALA and CALB) and Eversa Transform 2.0 have been immobilized on octyl- agarose beads at two different loads (1 mg/g and saturated support) and treated with phosphate and/or some metallic salts (Zn^{2+}, Co^{2+}, Cu^{2+}). They have been also immobilized on the support modified by the metallic phosphate, usually driving to biocatalyst with lower stability or marginal improvements. The effects of the phosphate/metal modification on enzyme features depended on the loading of the support. Some enzymes (TLL, CRL or CALA), mainly using the highly loaded biocatalysts, showed very significant improvement on enzyme stability after the treatment with some of the metal phosphates (next to a 20-fold factor), improvements that were not justified by the presence of metallic or phosphate ions in solution, as they had negative effects on enzyme stabilities. In some other cases, a significant increase in enzyme activity was detected (e.g., CALB). This could be explained by the modification of the nucleation places of the enzymes by the metallic phosphate, and this could help to explain the good results obtained in the nanoflower immobilization of many enzymes.

3.1 Introduction

Enzymes have many features that make them interesting as industrial biocatalysts, such as high activity at moderate temperature and pressure in aqueous media exhibiting a high specificity and selectivity (that prevents the production of undesired by-products) [1–7]. While the use of enzymes as industrial catalysts is spreading, the efforts to prevent the problems derived from their biological origin are also increasing. The huge advances in areas such as metagenomics [8–11], directed evolution [12,13] or site-directed mutagenesis [14] have permitted large leaps towards achieving enzymes with properties nearer to the required ones. One outstanding example of this potential is the production of enzymes bearing several active [15]. Among them, we can remark the so-called plurizymes [16] where a new biological but artificially designed active center is introduced in an enzyme and added to the natural enzyme active center and, after directed chemical modification with an organometallic catalyst on one

of these active centers, permitted to generate an enzyme molecule exhibiting catalytic activities far from the initial ones, and that had been used to perform cascade reactions [17].

Another critical point to be considered is the water-soluble character of enzymes, which makes their recovery and reuse difficult. This was the initial objective of enzyme immobilization, to permit the enzyme recovery and reuse [18]. Born from this necessity, many researchers tried to exploit the immobilization as a tool to improve other enzyme features. A proper immobilization may tune enzyme selectivity, specificity, inhibitions, even activity [19–22]. Moreover, it can be coupled to enzyme purification [23]. However, the enzyme feature that can be better improved using a rational immobilization strategy is enzyme stability [18,19,21,24–28]. The different causes that permit the increase of enzyme stability upon immobilization on pre-existing solids and how to maximize this have been recently reviewed [29].

Among the most popular strategies to immobilize-stabilize enzymes, the building of enzyme/metal nanoflowers, is attracting an increasing interest [30–35]. In this strategy, some specific place of the enzyme surface acts as nucleation points to form a metallic salt crystal surrounding the enzyme and giving a tridimensional structure with the form of petals of a flower, even a whole flower-like structure may be formed [30–36]. They usually give good values of activity and stability [37–41], but they have as a main problem its mechanical fragility. The nanoflowers are in some instances encapsulated in more rigid supports or are immobilized on magnetic materials, to facilitate the management of the biocatalyst and solve the mechanical fragility problems [42–48]. The good effects on enzyme features may be the result of some positive metal-enzyme interactions [49,50]. Some researches show that the structure and catalytic activity of nanoflowers may be conditioned by the protein morphology [51].

One alternative, that is proposed for first time in this paper, to take advantages of the stabilization effect of the enzyme immobilization via the nanoflowers building is to perform a similar treatment not to the free enzyme, but to previously immobilized enzymes, using a support already bearing mechanical positive properties. This will not permit the tridimensional growing of the nanoflower, but it may be possible that some positive effects can be reached if the enzyme molecules interact somehow with the metallic crystals. This may permit to couple the positive effects of enzyme stabilization via the enzyme immobilization on preexisting supports [18,19,21,24–28] and the positive effects of the nanoflowers [30–36], obtaining a stabilized biocatalyst with the desired mechanical properties.

That way, the objective of this paper is to analyze if it is possible to couple the positive effects of enzyme immobilization to the positive effects of enzyme modification with metal

phosphates. For this purpose, we have decided to utilize lipases immobilized on octyl agarose beads. This method involves the open form of the lipase [52] and it is able to immobilize-stabilize-hyperactivate-purify lipases [53]. Moreover, it is quite rapid, even more than enzyme diffusion, permitting a certain enzyme crowding that can affect the enzyme features [54–56] or being crosslinked using small bifunctional agents such as glutaraldehyde [57]. In this research, the support will be loaded with 10–20% of its maximal loading or with an excess of enzyme to ensure the full coating of the support surface with enzyme molecules. Different metallic salts, such as Cu^{2+}, Zn^{2+} and Co^{2+}, and diverse enzymes, such as the lipases from *Thermomyces lanuginosus* (TLL) [58], *Rhizomucor miehei* (RML) [59,60], Candida rugosa (CRL) [61–64], forms A and B of lipase from *Candida antarctica* (CALA and CALB) [65–70] and Eversa Transform 2.0 (EV), an industrial lipase preparation derived from TLL [71–73] have been utilized in this study. First, we have tried to build nanoflowers in solution, to determine conditions where these are formed, and these conditions were used to modify the enzymes immobilized on octyl agarose beads with the metallic phosphate crystals, determining the effects of different treatments on enzyme stability/activity.

3.2 Materials and Methods

3.2.1 Materials

In this study, we have employed different commercial lipase formulations. Most of them were supplied as liquid formulations and kindly donated by Novozymes Spain (Madrid, Spain): TLL with 20.77 mg protein/mL, Eversa® Transform 2.0 (EV) with 12.21 mg protein/mL, RML with 3.7 mg protein/mL, CALA and CALB presented 7.68 and 7.7 mg protein/mL, respectively. Lipase from Candida rugosa (CRL) was supplied from Sigma-Aldrich (St. Louis, MO, USA) as solid powder with 3.12 mg protein/g of powder. Bradford reagent (utilized to calculate the protein concentration [74]), *p*-nitrophenyl-butyrate (*p*-NPB), triacetin, cobalt chloride ($CoCl_2$), copper chloride ($CuCl_2$), and zinc chloride ($ZnCl_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Octyl Sepharose® CL-4B was acquired from GE Healthcare (Uppsala, Sweden). Purolite Lifetech® ECR8806F (methacrylate macroporous resin containing octadecyl - C18 - groups) (Purolite® C18) was kindly donated from Purolite® Ltd. (Wales, UK). All other reagents were of analytical grade.

3.2.2 Wetting of Purolite® C18 beads

Prior to use, dried Purolite® C18 beads were suspended in methanol (1:5, w/v) and kept under gentle stirring for 1 h to remove the air [75]. Subsequently, distilled water was added to obtain a water/methanol volumetric ratio of 1:1 (v/v) maintaining the stirring. After 15 min, the support was vacuum filtered and washed with distilled water (10 times with 10 volumes of water). Finally, the wet support was stored at 6–8 °C.

3.2.3 Immobilization of lipases

3.2.3.1 Immobilization of lipases on octyl-agarose beads

All lipases were immobilized by interfacial activation on octyl agarose beads using low (1 mg/g) and high enzyme loads (TLL: 20 mg/g; CALB: 25 mg/g; CALA: 20 mg/g; CRL: 30 mg/g; RML: 15 mg/g and EV: 20 mg/g) producing the biocatalysts: octyl-TLL, octyl-CALB, octyl-CALA, octyl-CRL, octyl-RML and octyl-EV, respectively [76,77]. Immobilization of lipases was performed diluting or dissolving the enzyme in 50 mL of 5 mM sodium phosphate at pH 7.0, followed by the addition of 5 g of support. The immobilization was conducted at room temperature under gentle stirring for 2 h. The enzyme activity in the supernatant, suspension and a reference (where octyl agarose was substituted by inert agarose) were quantified using *p*-NPB assay throughout the immobilization course. Afterwards, the suspensions were filtered and the biocatalysts were washed with distilled water, quantifying the enzyme activity using *p*-NPB as substrate, and stored at 4–6 °C.

3.2.3.2 Immobilization of lipases on wet Purolite® C18 beads

TLL was immobilized by interfacial activation in previously hydrated Purolite® C18 beads to produce Purolite®-TLL biocatalyst [75,78]. 50 mL enzymatic solution (2 mg/mL) prepared in 5 mM sodium phosphate were added to 5 g of support at pH 7.0. The immobilization process was conducted at room temperature under gentle stirring for 2 h, measuring the activity in suspension, supernatant and reference using *p*-NPB assay. Afterwards, the biocatalyst was filtered using a sintered filter funnel, washed with distilled water, quantifying the hydrolytic activity using triacetin as substrate, and stored at fridge.

3.2.4 Incubation of soluble TLL with zinc chloride and sodium phosphate buffer

The effects of $ZnCl_2$ concentration (8.84, 13.02, 17.03, 20.91, and 24.64 mM), sodium phosphate concentration (1, 5, 10, 15, 20, 25, 50, 100, and 150 mM), and NaCl concentration

(0, 25, 50, 75, 100, 125, 150, 175, and 200 mM) were investigated on the activity of the final TLL incorporated to the formed solid. Briefly, 800 μL of $ZnCl_2$ solution were added to 10 mL of 0.5 mg/mL soluble TLL solution in phosphate saline buffer (composed of the conditions to be optimized of NaCl and phosphate buffer) at pH 7.4. The incubation of the enzyme was conducted at room temperature under gentle stirring for 5 h, producing a solid biocatalyst and also some solid zinc phosphate precipitate. After the process, the biocatalysts were recovered by centrifugation at 4000*g* for 30 min at 4 °C. The precipitate was washed twice with distilled water, and finally resuspended in 5 mM sodium phosphate (pH 7.0). Measures of hydrolytic activities (*p*-NPB as substrate) in the initial enzyme solution and TLL organic-inorganic zinc phosphate biocatalyst (ZnP(TLL)-NFs) suspension were used to calculate the relative activity (ratio between the initial and final activity).

3.2.5 Incubation of immobilized enzymes with metallic chlorides and sodium phosphate

The modification of the support or immobilized enzyme was performed using the conditions where the highest activity of TLL was incorporated to the solid in Section 2.2.3. 800 μL of 230 mM metallic salt solution (17.03 mM of $ZnCl_2$, $CuCl_2$ or $CoCl_2$, final concentration) were added to 10 mL of 0.1 g/mL of support (octyl agarose beads) or immobilized enzyme in 10 mM of sodium phosphate/125 mM of NaCl buffer at pH 7.4. The modification was conducted at room temperature under gentle stirring for 5 h, producing modified octyl-agarose-enzyme or modified Purolite-enzyme. Afterwards, the modified support or the biocatalysts were filtered, washed with distilled water (10 times with 10 volumes of water), and stored at 4 °C. The modified supports were used to immobilize enzymes as described above for the unmodified ones and used as a reference of the effects of the support modification on the enzyme features. In some instances, the support or the immobilized enzymes were treated only with sodium phosphate buffer or only metallic salt under the same conditions described previously. The hydrolytic activity was quantified employing *p*-NPB as substrate for biocatalysts immobilized in octyl-agarose and triacetin for biocatalysts immobilized in Purolite®.

3.2.6 Thermal inactivation

In a standard experiment, 1 g of immobilized lipase biocatalyst was suspended in 10 mL of 10 mM Tris buffer at pH 7.0 and incubated at different temperatures. The temperatures were selected to have reliable but not too long half-lives of the unmodified immobilized enzymes (CALA: 85 °C; EV: 80 °C, TLL and CALB: 75 °C; CRL: 70 °C; RML: 60 °C). In some cases, 17.03 mM metallic chlorides were added to the inactivation medium to check their effects on

enzyme stability, or Tris buffer was replaced by 10 mM sodium phosphate buffer to evaluate the influence of phosphate on the stability of the modified and not modified biocatalysts [55]. Periodically, samples of 50 μL of inactivation suspension were collected to determine their residual activities. Residual activities were defined as the percentage of the biocatalysts initial activity maintained at the marked inactivation time. The experiment was performed employing *p*-NPB as substrate for biocatalysts immobilized in octyl-agarose and triacetin for biocatalysts immobilized in Purolite®.

3.2.7 Standard activity assay

One unit of activity (U) was defined as the amount of enzyme that hydrolyzes one μmol of substrate per minute under the described conditions.

3.2.7.1 Hydrolysis of p-NPB

The hydrolytic activity using *p*-NPB as substrate was determined by spectrophotometry, following the increase in the absorbance at 348 nm due to *p*-nitrophenol (*p*-NP) (isosbestic point of *p*-NP) release in the medium, using a thermostatization system at 25 °C, (molar extinction coefficient, ε = 5150 M^{-1} cm^{-1}) under magnetic stirring for 1.5 min [79]. Briefly, 50 μL of soluble or immobilized enzyme sample was added into a mixture of 2.5 mL of 25 mM sodium phosphate at pH 7.0. Then, 50 μL of 50 mM *p*-NPB dissolved in acetonitrile were added to start the reaction.

3.2.7.2 Hydrolysis of triacetin

Samples of 300 μL of immobilized enzyme (125 mg/mL) were added to 3 mL of 50 mM of triacetin prepared in 50 mM sodium phosphate buffer at pH 7.0. Hydrolysis was carried out at 25 °C under magnetic stirring for 10 min. The hydrolytic activity in triacetin was quantified by the detection of diacetin. The degree of conversion was calculated by High Performance Liquid Cromatography (HPLC) in a Waters 486 chromatograph (Waters, Millford, USA) equipped with a UV/VIS detector (set to 230 nm) using a Kromasil C18 column (15 cm × 0.46 cm) with a mobile phase composed of 15% (*v*/v) of water and 85% (v/v) of acetonitrile with a flow rate of 1 mL/min. Although only 1,2 diacetin was enzymatically produced, at pH 7.0 the acyl migration favored the existence of a mixture of 1,2 and 1,3 diacetin [80]. To avoid that the reaction could progress further (to monoacetins), the enzyme activity was calculated using triacetin conversions between 15 and 20%. The retention times were 4 min for both 1,2 and 1,3 diacetins and 18 min for triacetin [81].

3.3 Results and Discussion

3.3.1 Determination of conditions where TLL is immobilized in metallic phosphate precipitate

First of all, we assayed the immobilization of free TLL forming nanoflowers under different conditions, altering the concentration of the metal chloride, sodium phosphate and NaCl, variables described in the literature as important to get enzyme/metal hybrid nanoflowers [36,82–84]. The objective was not to optimize the preparation of the nanoflowers, but just to ensure conditions where the enzyme immobilization occurred on the metal phosphate crystal. We found that all these parameters affected the final activity of the immobilized TLL, with the final activity being lower when using the lowest used concentrations and decreasing after reaching a maximum. Figure A3.1 shows a model situation using $ZnCl_2$, where the optimal activity retention on the precipitate was achieved using 17.03 mM $ZnCl_2$, 10 mM sodium phosphate and 125 mM NaCl. Similar optimal conditions were found using the other metals although with different activity retentions (results not shown). That way, we decided to employ these conditions in the modification of the immobilized TLL.

3.3.2 Preparation of immobilized TLL preparations

As explained in the Methods section, in this research we have prepared immobilized enzymes at two very different loadings, one far from the maximum loading of the support (1 mg/g) and other exceeding the loading capacity of the support. Figure A3.2 shows the immobilization courses for both TLL preparations. Using 1 mg/g, all enzyme activity was immobilized in the first activity determination and the activity increase to more than double. Using an excess of enzyme, the immobilization yield was only around 50%, but even under those conditions, the suspension immobilization increased the activity to 180%. This increase on enzyme activity upon immobilization is related to the stabilization of the open form of the enzyme [52] and the prevention of the formation of enzyme-enzyme dimers [85] (more favored when the enzyme was at higher concentrations). After washing, the lowly loaded enzyme presented an activity around 3-fold lower than the highly loaded biocatalyst (Table 3.1), although the load is 10-fold lower (Figure A3.2). This may be caused by the much higher diffusional limitations for the higher loaded and more active preparation [54,56]. That way, we have prepared a biocatalyst with the support surface fully coated with TLL molecules, and another one with a much lower load.

3.3.3 Incubation of immobilized TLL with metal and phosphate

In all cases, all TLL activity remained attached to the support, the treatment does not produce any enzyme release. Table 3.1 shows the effects of the metals and phosphate treatments of both immobilized TLL preparations on the enzyme activity. Using phosphate or metallic chlorides individually, the enzyme activity hardly changed. Moreover, the biocatalysts remained with the white color typical of agarose beads. However, when incubating the immobilized enzyme with phosphate and metallic chlorides, the activity of both biocatalysts showed a significant decrease, sharper for the highly loaded biocatalyst, except in the case of $ZnCl_2$. In this instance, the lowly loaded biocatalyst maintained the activity (even results shown a slight increase in activity) and the highly loaded biocatalyst decreased the activity by around 21%. Similar results were also found in the free TLL biomineralization process for the formation of nanoflowers [36]. In this case, the authors showed that the different metal ions in solution did not affect the specific activity of the biocatalyst, however, their corresponding metal salt/sodium phosphate in mineral form were responsible for modifying the catalytic properties. Furthermore, it can be expected that the effect of the modification of the enzyme by the metal phosphates may be different using different immobilization protocols, as it has been described for other lipase chemical or physical modifications [86–89]. Ongoing experiments in our laboratory show that the effects of the modification with metal phosphate of immobilized enzymes may also depend on the immobilization protocol (unpublished results).

The color change in the biocatalyst (Figure A3.3) also suggests that substrate diffusional problems might occur due to the formation of metallic phosphate structures within the biocatalyst particles. Similar colors of the particles could also be found when using just octyl agarose (Figure A3.4). That way, as a control, we prepared a support exposed to the metal and the phosphate, and TLL was immobilized on it. The biocatalysts presented a slightly lower activity than using the unmodified agarose, not very different to those where the enzyme is modified after the immobilization (Table 3.2).

Moreover, we tried to immobilize more enzyme on the support fully coated with TLL and treated with the metal phosphate. Using 50 mM of buffer to prevent the immobilization on the previously immobilized enzyme (very likely via ion exchange) [90], there was not immobilization of TLL, suggesting that the enzyme was immobilized directly on the support surface via interfacial activation.

Next, all the biocatalysts were inactivated in 10 mM Tris at pH 7 and 75 °C and compared to the original octyl-TLL biocatalyst (Figure 3.1). The highly loaded biocatalyst had a higher apparent stability than the lowly loaded biocatalyst. This presented a moderate

magnitude that can be justified by the substrate diffusional matters, which reduce the detected initial activity in the highly loaded biocatalyst and can produce “apparent” stabilizations [54,56]. Using the lowly loaded biocatalyst, the previous incubation of the immobilized enzyme with phosphate has no effect on enzyme stability. The incubation of the biocatalyst on $ZnCl_2$/Tris before the inactivation produced a moderate enzyme stabilization, similar to the results obtained when immobilizing the enzymes on the support previously treated with $ZnCl_2$/sodium phosphate. The most stable preparation was those prepared by incubation of the immobilized enzyme with $ZnCl_2$/sodium phosphate: it maintained over 40% of the activity after 2 h of incubation, while the unmodified enzyme reached this activity value after only 0.5 h.

Table 3.1. Effect of the treatment with phosphate, chloride metallic salt and metallic salt/phosphate on the specific activity of lowly and highly loaded octyl-TLL biocatalysts. The hydrolytic activity was measured employing 1 mM *p*-NPB at pH 7.0 and 25 °C. Experiments were performed as described in Methods.

Enzyme load	Activity with *p*-NPB (U/g)							
	Treatment							
	No treatament	Phosphate	$ZnCl_2$	$CuCl_2$	$CoCl_2$	$ZnCl_2$/phosphate	$CuCl_2$/phosphate	$CoCl_2$/phosphate
1 mg/g	84.79 ± 5.57	83.20±4.51	87.03 ± 2.35	86.32 ± 3.30	81.25 ± 3.06	87.32 ± 4.37	64.77 ± 2.23	70.80 ± 3.50
20 mg/g	256.38 ± 4.19	247.54 ± 10.93	250.87 ± 11.54	248.45 ± 9.42	282.37 ± 12.12	202.98 ± 9.15	150.90 ± 7.55	143.03 ± 3.15

Table 3.2. Hydrolytic activities of TLL immobilized on octyl agarose previously modified with $ZnCl_2$/sodium phosphate (octyl-ZnP-TLL), $CuCl_2$/sodium phosphate (octyl-CuP-TLL) and $CoCl_2$/sodium phosphate (octyl-CoP-TLL). The hydrolytic activity was measured using 1 mM *p*-NPB at pH 7.0 and 25 °C. Experiments were conducted as described in the Methods.

Enzyme load	Activity with *p*-NPB (U/g)			
	Octyl-TLL	Octyl-ZnP-TLL	Octyl-CuP-TLL	Octyl-CoP-TLL
1 mg/g	72.76 ± 2.91	64.22 ± 2.58	70.64 ± 4.40	74.30 ± 1.85
20 mg/g	286.56 ± 16.24	241.62 ± 1.76	275.85 ± 6.45	246.62 ± 15.36

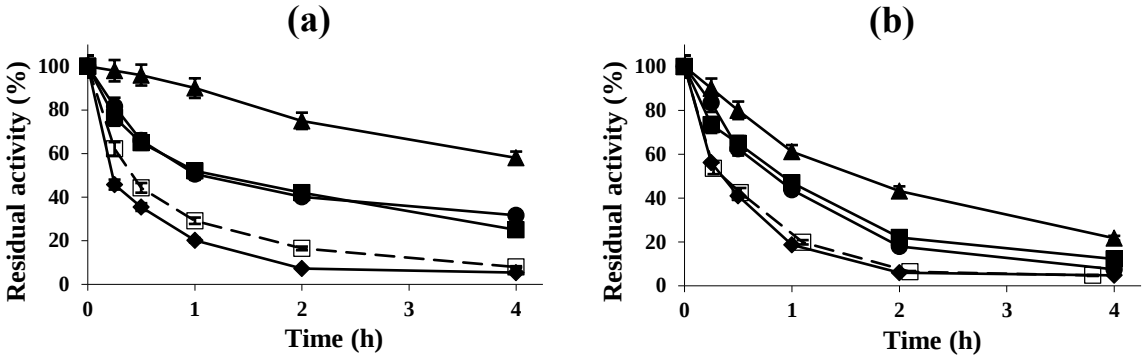

Figure 3.1. Inactivation course of untreated and treated octyl-TLL prepared at two loadings (a) 1 mg/g and (b) 20 mg/g. The biocatalysts were inactivated in 10 mM Tris at pH 7 and 75 °C. Other specifications are described in Methods. Untreated biocatalysts (open squares and dotted line); biocatalyst treated with phosphate (rhombus); $ZnCl_2$ (circles); $ZnCl_2$/sodium phosphate (triangles) and TLL immobilized on octyl previously modified with $ZnCl_2$/sodium phosphate (squares).

Using the highly loaded biocatalyst, the previous incubation in sodium phosphate had a slightly negative effect on enzyme stability. The incubation of the immobilized enzyme in $ZnCl_2$/Tris produced an enzyme stabilization similar to that found when using a support pretreated with $ZnCl_2$/sodium phosphate to immobilize TLL. The highest stabilization was again found treating the immobilized enzyme with $ZnCl_2$/sodium phosphate. Using the highly loaded biocatalyst, stabilizations were more relevant, now, the enzyme treated with $ZnCl_2$/sodium phosphate maintained almost 60% of the initial activity after 4 h, similar to that maintained by the untreated enzyme after only 15 min.

To discriminate if this stabilizing effect could be consequence of the release of Zn^{2+} or phosphate during the inactivation, the inactivation was also performed in the presence of these compounds. Figure A3.5a shows that the inactivation of octyl-TLL at low loading in the presence of free Zn^{2+} was very similar to the inactivation in the absence of this metal, while using the high loading the stability was even slightly reduced. Using the Zn^{2+}/sodium phosphate treated preparations, the presence of free metal was slightly negative for the stability of both, highly and lowly loaded biocatalysts. This suggested that the direct interaction with the metal is not the reason for enzyme stabilization. Figure A3.5b shows that the presence of 10 mM sodium phosphate presented a negative effect on the stability of octyl-TLL, more significant using the highly loaded biocatalyst. These negative effects were much clearer when the Zn^{2+}/sodium phosphate-treated preparations were used, that become even less stable than the

not treated enzymes. This negative effect of phosphate ions on the stability of lipases immobilized on octyl-agarose had been previously reported, but using higher concentrations [91,92].

The possibility of releasing the enzyme from the support before and after metal phosphate treatment was assayed by incubation of the biocatalysts in 1% Triton X-100. In both cases most enzyme molecules were released to the supernatant, as verified by activity determinations and SDS-PAGE experiments (not shown results), suggesting that the enzymes were not really forming multi-molecular structure as it is supposed to be in the case of standard nanoflowers.

Next, the stability of immobilized TLL modified with the other metals and phosphate was studied (Figure A3.6). Using only Cu^{2+}, the stabilities of both highly and lowly loaded TLL biocatalyst marginally decreased after the modification. However, using Co^{2+}, the modified biocatalyst increased the stability, more using the lowly loaded biocatalyst than using the highly loaded biocatalyst, making the inactivation courses of both modified biocatalysts almost identical. However, the stabilization was not as significant as in the case of Zn^{2+}. These results suggest that the binding/nucleation sites in the protein or the effect of being modified by the different metals are distinct for Zn^{2+}, Cu^{2+}, Co^{2+}, and, consequently, the mineral form of these ions led to the formation of a conformational structure with different physicochemical properties [36].

To check if the treatment with metal phosphate of the support before immobilizing the enzyme could hinder the effect of treating the enzyme with another metal phosphate after its immobilization, we utilized a support previously modified with $CoCl_2$/sodium phosphate to immobilize TLL. This biocatalyst presented properties not very different to those of the enzyme immobilized on octyl agarose, and their color could make easy to determinate if the new metal phosphate was or not incorporated to the support. That way, the enzyme was immobilized on octyl-agarose previously modified with $CoCl_2$/sodium phosphate, and later, the immobilized enzyme was also modified, with the three metals and phosphate. Table A3.1 shows that the further modification of the lowly loaded biocatalyst with sodium phosphate and the different metal chlorides produced a slight increase on enzyme activity, except using Cu^{2+}. Using the highly loaded biocatalyst, the increase in activity is more significant, although did not exceed a 25%. As shown in Figure A3.7, this treatment produced effects on enzyme stability, qualitatively similar to those where the enzyme was immobilized on the unmodified support. The modification with $ZnCl_2$/sodium phosphate produced a great stabilization for both enzyme biocatalysts prepared in $CoCl_2$/sodium phosphate modified support. Figure A3.8 shows from

the color of the biocatalyst how the new metal can become incorporated to the support, and apparently the previous one has not a relevant influence on the final stability of the biocatalysts.

That way, it is necessary to modify the immobilized enzyme with the metal and phosphate to have the optimal stabilization result.

3.3.4 Modification with Zn^{2+}/phosphate of TLL immobilized on Purolite

To determine the importance of the support in the results, TLL was immobilized on Purolite and the immobilized enzyme was incubated in Zn^{2+}/phosphate. The biocatalyst took the color of the corresponding metal (Figure A3.9). Then, its stability was determined by incubating the biocatalysts at 75 °C and pH 7 (Figure 3.2). To make a fair comparison, we also inactivated the octyl preparations using triacetin as substrate, as it has been shown that the inactivation may follow a different trend depending on the substrate [81]. The enzyme immobilized on Purolite was much more stable than that immobilized on octyl-agarose, and interestingly, the stabilization achieved by the metal/phosphate treatment was similar to effects on TLL immobilized on octyl-agarose.

That way, the stabilizing effects of the treatment could be extrapolated to other supports and were not restricted to octyl-agarose beads.

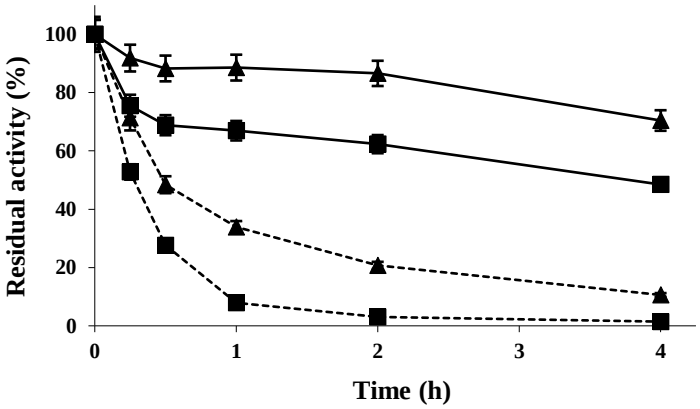

Figure 3.2. Inactivation course of octyl-TLL (sotted lines) and Purolite-TLL (solid lines) modified with $ZnCl_2$/sodium phosphate. The biocatalysts were inactivated at 75 °C, in presence of 10 mM Tris buffer at pH 7.0. Other specifications are described in Methods. The hydrolytic activity to determine residual activity was measured using 50 mM triacetin at pH 7.0 and 25 °C. Solid squares: octyl-TLL or Purolite-TLL; solid triangles: octyl-TLL or Purolite-TLL modified with $ZnCl_2$/sodium phosphate.

3.3.5 Modification with different metal chlorides and phosphate of the other immobilized enzymes

Figure A3.10 shows the immobilization of the different enzymes on octyl agarose at high and low loadings. Using low loadings, except for CALB (and EV, where the hyperactivation is minimal), the immobilized biocatalysts present higher activities that the corresponding free counterparts. Using the highly loaded biocatalyst, many enzymes had a significant decrease in their activity after immobilization, and in all cases some enzyme activity remains in the supernatant after immobilization, ensuring that the whole support surface is coated with enzyme molecules. The decrease in activity using the highly loaded biocatalyst is very likely due to substrate diffusion matters [54,56].

Table 3.3 shows the activities of the different biocatalyst after being modified with metal chloride/phosphate while Table 3.4 shows the activities of the enzymes immobilized on the metal/phosphate modified supports.

Using CALB, the lowly loaded biocatalyst activity slightly increased after the modification, depending on the used metal, while the highly loaded biocatalyst maintains the activity using Zn^{2+} and decreased using the other metals. The situation is fully different when the enzyme is immobilized on modified supports. While using the low loaded biocatalyst the activity slightly decreased compared to that of the unmodified support, using the highly loaded biocatalyst there was a significant increase in activity (by more than 50%), in contraposition with the activity decrease observed when the enzyme was modified with the metals.

Using CALA, the modification of the immobilized enzyme promoted a slight decrease in the enzyme activity using the lowly loaded biocatalyst, except when using Co^{2+} that produced a 15% increase in enzyme activity. The highly loaded biocatalyst almost maintained its activity unaltered after the different modifications. When the enzyme was immobilized on the modified support, the results were very different. The activity decreased more for the highly loaded biocatalyst (by more than one third using Co^{2+}) than for the lowly loaded one (that almost maintained intact the activity).

Using CRL, the treatment of the biocatalysts increased the activity after modification, being the most active one the lowly loaded biocatalyst that modified with Co^{2+} (almost increased the activity by 30%), while for the highly loaded biocatalyst the highest activity was obtained using Cu^{2+} (a hyperactivation of around 15%). When the enzyme was immobilized on modified supports, Cu^{2+} increased the activity using both loadings (by 10%). The other modified supports increased in a very low ratio the activity of the low loaded biocatalyst, while decreased the activity of the highly loaded one (by 20% using Co^{2+}).

Table 3.3. Effect of the treatment with metallic chloride salts/sodium phosphate on the specific activity of octyl-TLL biocatalyst using 1 mM *p*-NPB at pH 7.0 and 25 °C. Experiments were conducted as described in Methods.

Biocatalysts	Activity with *p*-NPB (U/g)							
	Without modification		Modification using $ZnCl_2$/sodium phosphate		Modification using $CuCl_2$/sodium phosphate		Modification using $CoCl_2$/sodium phosphate	
	1mg/g	20 mg/g	1mg/g	20 mg/g	1mg/g	20 mg/g	1mg/g	20 mg/g
Octil-CALB	17.12 ± 0.85	293.48 ± 13.87	19.41 ± 1.97	298.97 ± 11.95	17.44 ± 0.67	246.63 ± 12.33	18.59 ± 0.72	254.60 ± 12.56
Octil-CALA	103.99 ± 5.20	164.58 ± 9.22	95.39 ± 3.77	166.90 ± 7.34	96.07 ± 3.80	172.26 ± 5.61	120.17 ± 6.90	179.02 ± 7.54
Octil-CRL	94.90 ± 4.75	153.58 ± 7.67	113.40 ± 6.67	168.82 ± 5.44	112.90 ± 6.64	175.39 ± 6.76	122.49 ± 6,21	159.99 ± 8.21
Octil-RML	50.17 ± 2.51	73.33 ± 4.67	51.19 ± 2.56	68.95 ± 2.55	48.03 ± 2.41	55.90 ± 2.56	54.56 ± 1.72	75.93 ± 1.67
Octil-EV	111.71 ± 5.89	195.02 ± 10.05	119.20 ± 6.96	190.56 ± 8.56	105.93 ± 2.29	186.84 ± 9.89	98.84 ± 2.94	179.93 ± 9.89

Table 3.4. Hydrolytic activities of different lipases immobilized on octyl agarose modified with metallic salt/sodium phosphate. The hydrolytic activity was measured utilizing 1 mM *p*-NPB at pH 7.0 and 25 °C. Experiments were conducted as described in the Methods.

Biocatalysts	Activity with *p*-NPB (U/g)							
	Octyl		Octyl-ZnP		Octyl-CuP		Octyl-CoP	
	1mg/g	20 mg/g	1mg/g	20 mg/g	1mg/g	20 mg/g	1mg/g	20 mg/g
CALB	26.06 ± 1.4	288.37 ± 11.35	21.54 ± 0.86	378.99 ± 0.52	20.94 ± 1.30	403.68 ± 18.89	21.02 ± 0.52	403.33 ± 25.12
CALA	108.25 ± 4.22	229.37 ± 13.57	104.21 ± 4,19	177.30 ± 2.67	107.04 ± 6.66	164.42 ± 3.85	105.52 ± 2.64	143.26 ± 9.92
CRL	103.57 ± 4.12	167.91 ± 9.52	110.90 ± 4.46	153.37 ± 3.02	115.95 ± 7.53	184.07 ± 4.31	110.38 ± 2.67	132.78 ± 8.27
RML	63.08 ± 2.25	97.89 ± 5.55	58.64 ± 2.35	96.70 ± 1.22	49.19 ± 3.06	101.59 ± 2.37	56.67 ± 1.42	92.25 ± 5.56
EV	122.30 ± 4.89	212.54 ± 11.05	155.66 ± 6.26	224.46 ± 4.46	178.80 ± 11.13	225.01 ± 5.30	158.91 ± 3.97	240.77 ± 14.01

The metal phosphate treatment of immobilized RML had low effects on enzyme activity, increasing the activity by less than 10% using the lowly loaded biocatalyst. The highly loaded biocatalyst final activities depended on the metal; using Co^{2+} there is a small increase, while Zn^{2+} and Cu^{2+} promoted a decrease in enzyme activity (by 25% in the last case). When immobilized on modified supports, RML decreased the activity using the lowly loaded biocatalyst (almost to 75% using Cu^{2+}) and remained similar using the highly loaded biocatalysts.

Finally, the modification of lowly loaded EV biocatalyst produced small effects on the enzyme activity, in some instances this effect was negative, in some positive, while the highly loaded biocatalyst slightly decreased the activity in all instances (by a maximum lower than 10% using Co^{2+}). Results were very different using the modified supports to immobilize the enzyme, as this increased the activity using both loadings, by a 50% using Cu^{2+} using the lowly loaded biocatalyst.

That is, the metal phosphate modification has very diverse effects on the activities of the different biocatalysts, and this is not directly related to the loading of the biocatalyst (that could permit that the metal can influence the intermolecular interactions). It can be neither explained by the presence of metal phosphate on the support, as this has frequently an effect on the enzyme activity opposite to that of the modification of the immobilized enzyme. Probably, the effects caused by the mineral form of Zn^{2+}, Cu^{2+}, Co^{2+} can be attributed to the binding/nucleation site present in the regions of the lipases used. When the nucleation point was located in the vicinity of the active site or in the regions that allow the movement of the "cap" domain, the enzyme structure suffered conformational distortions that compromised the physicochemical properties. However, when the nucleation points did not involve key regions of the enzyme structure, important characteristics were added to the biocatalyst [36].

However, our main interest in this paper is to analyze the effects of the different treatments on the final enzyme stability. We include the results using the support modified by the metallic phosphate, that is the real reference to check the effect of the enzyme modification (as this support modification is unavoidable when modifying the immobilized enzyme), and the unmodified enzyme and support, that is the biocatalyst whose stability we intend to improve.

Starting with CALB, Figure 3.3 shows that the highly loaded biocatalyst is less stable than the lowly loaded one, as previously reported by Fernandez-Lopez et al. [54]. The modification of the biocatalysts with Zn^{2+}/phosphate presented a marginal negative effect on the enzyme stability, similar to the immobilization of the enzyme in the modified support. Modifying the lowly loaded biocatalyst with Cu^{2+}/phosphate, the enzyme stability marginally

increased, while the immobilization of the enzyme in the modified support presented a marginal negative effect. However, using the highly loaded biocatalyst, the effects on the enzyme stability of this treatment are negative, and much more if the enzyme was immobilized on the modified support. That is, while the modification of the enzyme seems to be positive, the modification of the support is clearly negative for the enzyme stability. Finally, the modification with Co^{2+}/phosphate produced a decrease in enzyme stability for the lowly loaded biocatalyst, again more significant using the modified support that modifying enzyme and support. The enzyme immobilized on the modified support at low load became more unstable than the highly loaded biocatalyst. Using the highly loaded biocatalyst, the negative effects were similar when modifying the immobilized enzyme or immobilizing the enzyme in the modified support.

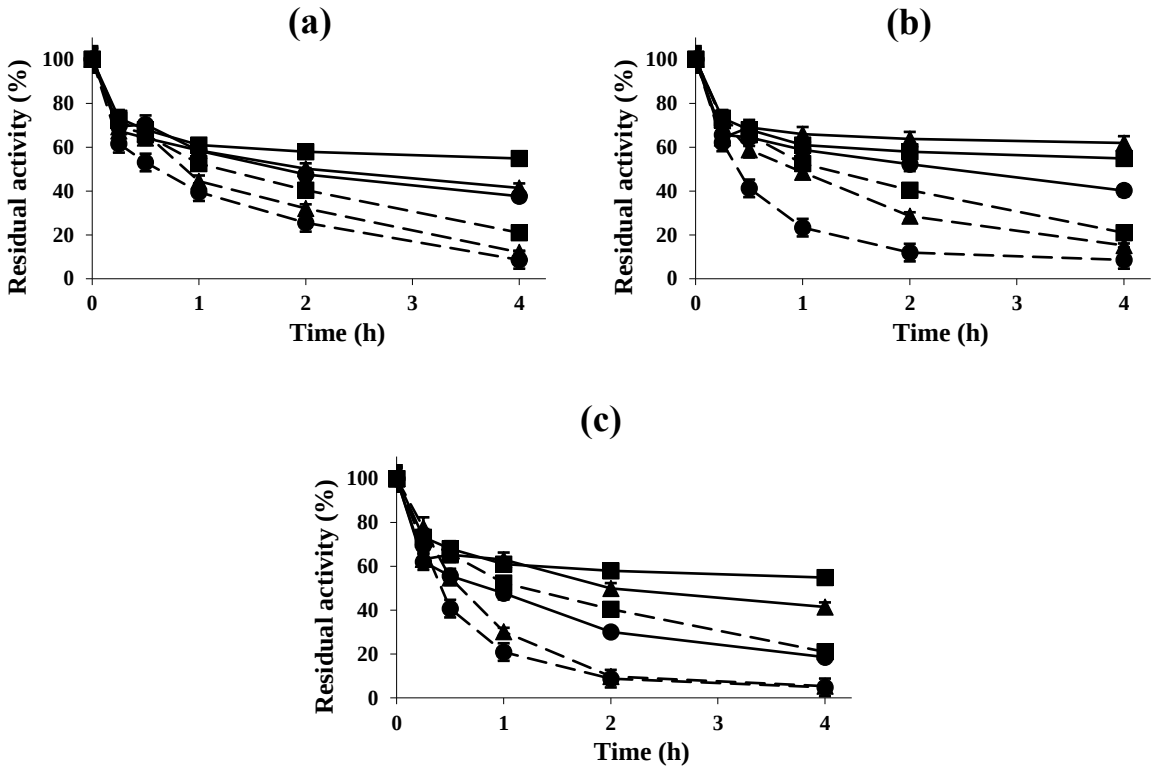

Figure 3.3. Inactivation courses of different octyl-CALB biocatalysts in 10 mM Tris buffer at pH 7.0 and 75 °C. Other specifications are described in Methods. (a) Modified with $ZnCl_2$/sodium phosphate, (b) Modified with $CuCl_2$/sodium phosphate, and (c) Modified with $CoCl_2$/sodium phosphate. The solid and dashed lines correspond to protein loads of 1 mg/g and 25 mg/g, respectively. Squares: unmodified octyl-CALB; triangles: modified octyl-CALB; circles: CALB immobilized on octyl agarose modified with metallic phosphate.

Figure 3.4 shows the results using CALA. In this instance, the highly loaded biocatalyst had a higher stability than the lowly loaded one. The modification (or the immobilization on modified support) with Zn^{2+}/phosphate gave a similar lower stability for the lowly loaded biocatalyst. Using the highly loaded biocatalysts, the immobilization on the modified support is significantly negative for the enzyme stability, while the stability increased if the enzyme is modified after immobilization. This means that the stabilization achieved modifying the enzyme is very significant. If the modification is performed using Cu^{2+}/phosphate, both biocatalysts appreciably decreased their stability by immobilizing on the modified support, and less if being modified after enzyme immobilization. Using Co^{2+}/phosphate, the effects were slightly negative using the lowly loaded biocatalyst in when immobilizing on modified supports or modifying the immobilized enzyme, the effect were much smaller using the highly loaded biocatalyst, which even is slightly more stable after modifying the immobilized enzyme than without this treatment.

To check if the positive effect of Zn^{2+}/phosphate on the enzyme stability was caused by the free component of the salt, the enzyme was inactivated in the presence of $ZnCl_2$ and sodium phosphate (Figure A3.11). In this instance, Zn^{2+} presented a clear destabilization effect for octyl-CALA at both loads. This very negative effect of the presence of free Zn^{2+} is even more drastic for the Zn^{2+}/phosphate treated lowly loaded biocatalyst, while it had a much smaller impact on the enzyme stability suing the highly loaded and modified biocatalyst. That way, the presence of free Zn^{2+} cannot explain the stabilization found using the metallic phosphate modification. Phosphate presented a very negative effect on the enzyme stability of the lowly loaded octyl-CALA, smaller using the highly loaded biocatalyst, as previously reported by Kornecki et al. and Zaak et al. [91,92]. After the modification, this negative effect of phosphate was accentuated (the stability of both, modified and not modified highly loaded biocatalyst become identical). That way, the stabilization found by modification with phosphate and zinc chloride cannot be explained by the presence of these agents in solution, as both had negative effects on enzyme stability. The treatment of the immobilized enzymes only with phosphate or the zinc chloride did not present any significant effect on enzyme activity/stability (Table A3.2 and Figure A3.12).

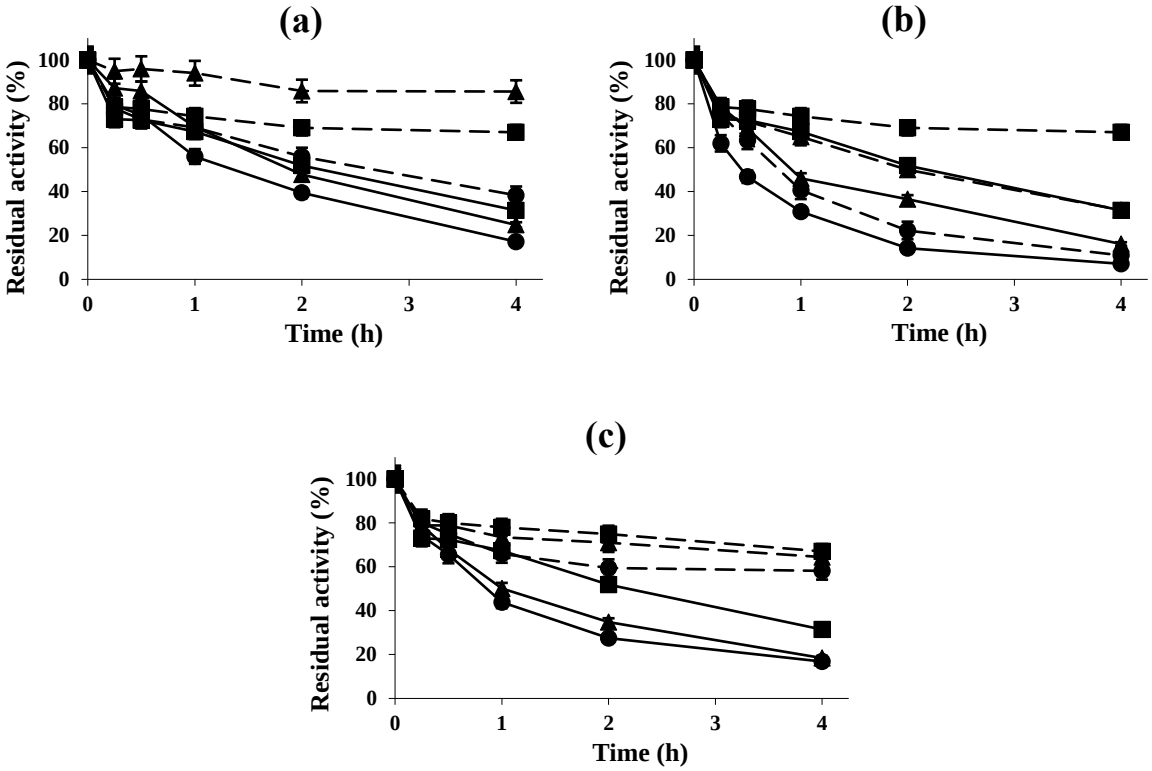

Figure 3.4. Inactivation courses of different octyl-CALA biocatalysts in 10 mM Tris buffer at pH 7.0 and 85 °C. Other specifications are described in Methods. (a) Modified with $ZnCl_2$/sodium phosphate, (b) Modified with $CuCl_2$/sodium phosphate, and (c) Modified with $CoCl_2$/sodium phosphate. The solid and dashed lines correspond to protein load of 1 mg/g and 20 mg/g, respectively. Squares: unmodified octyl-CALA; triangles: modified octyl-CALA; circles: CALA immobilized on octyl-agarose modified with the metallic phosphate.

Figure 3.5 shows that CRL stability was very similar using low or high loaded biocatalysts. The lowly loaded biocatalyst slightly decreased the stability when using Zn^{2+}/phosphate before or after immobilization. This is very different to the results using highly load biocatalysts. While the immobilization on the modified support gave very similar stability to that on the no modified support, the modification of the immobilized enzyme permitted to reach very high stabilization factors (e.g., after 1 h the modified enzyme maintains almost 55% of the initial activity, while the unmodified one maintains only 8%). Figure A3.13 shows that the presence of Zn^{2+} produced a small decrease in the stability of octyl-CRL biocatalysts, and this effect was much more evident when using the jiggly loaded biocatalyst modified with Zn^{2+}/phosphate, in the presence of the metal salt the stabilization achieved by the treatment of the immobilized enzyme with Zn^{2+}/phosphate almost disappeared. Also, phosphate produced a

negative effect on enzyme stability, almost eliminating the stabilization achieved by the modification of the immobilized enzymes. Again, it is not the effect of the free components of the modification the responsible of the stabilization, but the interaction with the metal phosphate. Figure 3.5 shows that similar stabilization could be achieved modifying octyl-CRL with Cu^{2+}/phosphate, and slightly lower stabilization using Co^{2+}/phosphate (maintaining more than 35% after 1 h of inactivation). Again, the treatment of the immobilized enzymes only with phosphate or the zinc chloride did not present any significant effect on enzyme activity/stability (Table A3.3 and Figure A3.14).

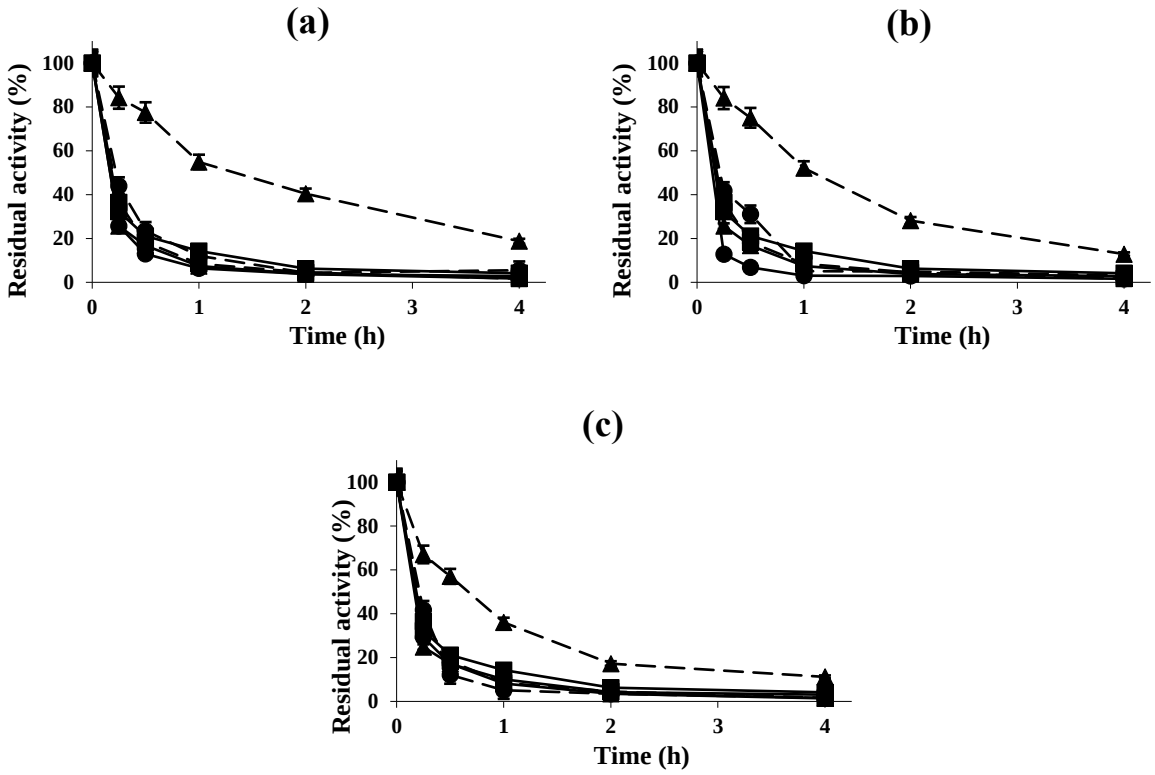

Figure 3.5. Inactivation courses of different octyl-CRL biocatalysts in 10 mM Tris buffer at pH 7.0 and 70 °C. Other specifications are described in Methods. (a) Treated with $ZnCl_2$/sodium phosphate, (b) Treated with $CuCl_2$/sodium phosphate, and (c) Treated with $CoCl_2$/sodium phosphate. The solid and dashed lines correspond to protein load of 1 mg/g and 30 mg/g, respectively. Squares: unmodified octyl-CRL; triangles: modified octyl-CRL; circles: CRL immobilized in octyl-agarose modified with metallic phosphates.

Using RML (Figure 3.6), the highly loaded biocatalyst showed a better stability than the low loaded biocatalyst. The latter one maintains its stability when incubated with

Zn^{2+}/phosphate, while its stability decreased when it is immobilized on the modified support. That way, the modification of the enzyme somehow reversed the negative effect of the modification of the support. This is not visualized using the highly loaded biocatalyst, the modification of the immobilized enzyme or the immobilization on the modified support produce a similar small negative effect on the enzyme stability. The negative effect on the enzyme modification or the immobilization on the modified support is more evident using Cu^{2+}/phosphate for both, low and high loads. The use of Co^{2+}/phosphate gave results similar to the use of Zn^{2+}/phosphate.

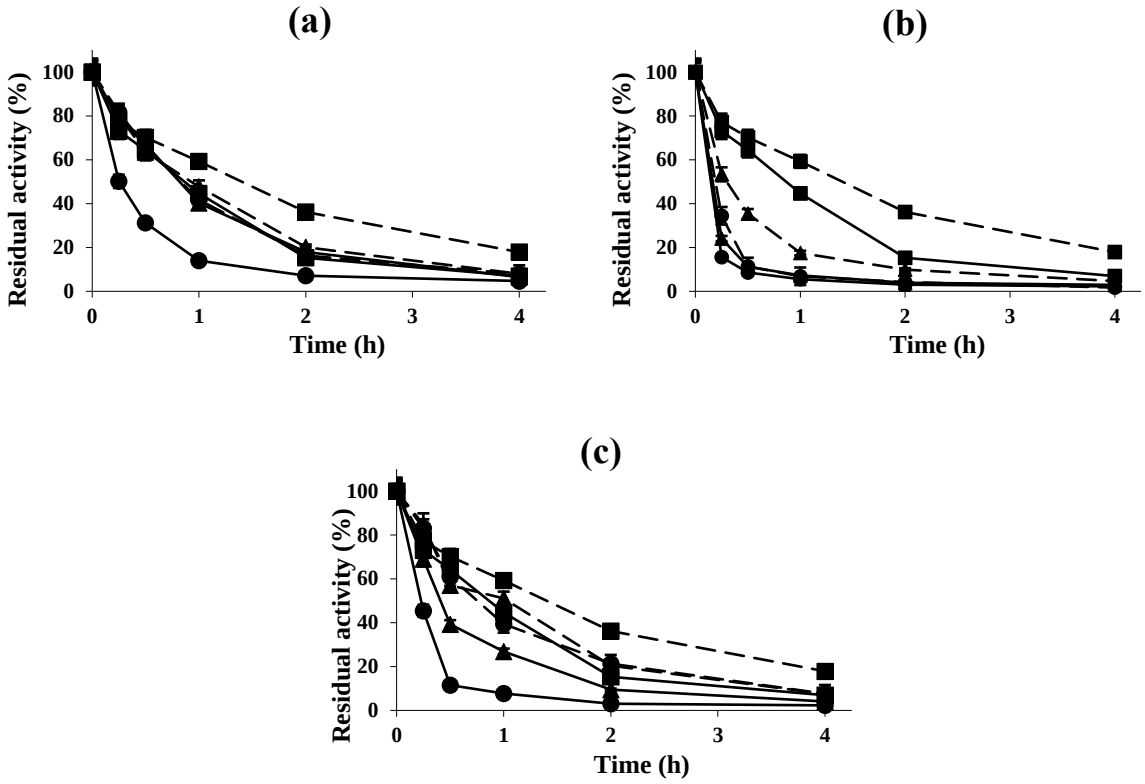

Figure 3.6. Inactivation courses of different octyl-RML biocatalysts in 10 mM Tris buffer at pH 7.0 and 60 °C. Other specifications are described in Methods. (a) Treated with $ZnCl_2$/sodium phosphate, (b) Treated with $CuCl_2$/sodium phosphate, and (c) Treated with $CoCl_2$/sodium phosphate. The solid and dashed lines correspond to protein loads of 1 mg/g and 15 mg/g, respectively. Squares: unmodified octyl-RML; triangles: metallic phosphate octyl-RML; circles: RML immobilized on metallic phosphate modified octyl-agarose beads.

Figure 3.7 shows the results obtained using EV. This enzyme is industrially evolved enzyme from TLL, however the results are very different to the ones obtained using TLL. The

stabilities of high and low loaded biocatalysts are almost identical. The use of Zn^{2+}/phosphate and Co^{2+}/phosphate almost did not produce changes in enzyme stability, perhaps we can remark the destabilization of EV immobilized on the support modified with the metal phosphate at high enzyme load, while Cu^{2+}/phosphate presented much more negative effects on enzyme stability.

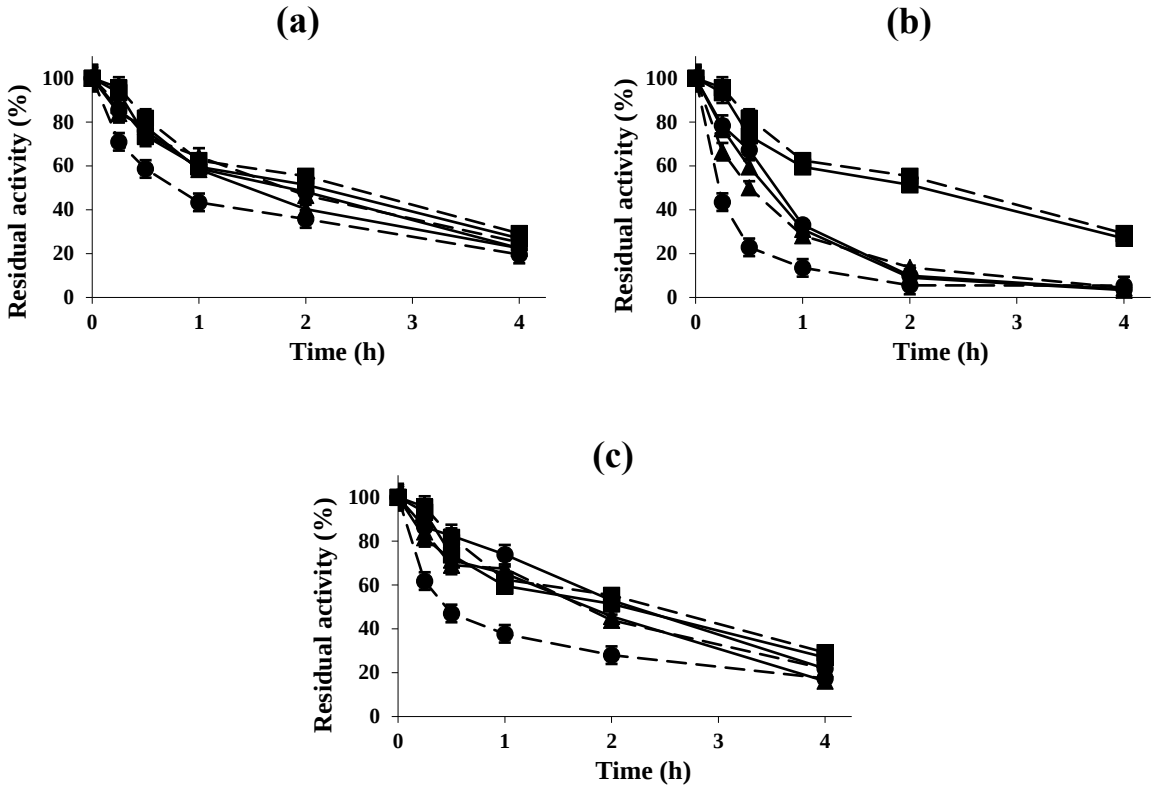

Figure 3.7. Inactivation courses of different octyl-EV biocatalysts in 10 mM Tris buffer at pH 7.0 and 80 °C. Other specifications are described in Methods. (a) Treated with $ZnCl_2$/sodium phosphate, (b) Treated with $CuCl_2$/sodium phosphate, and (c) Treated with $CoCl_2$/sodium phosphate. The solid and dashed lines correspond to protein loads of 1 mg/g and 20 mg/g, respectively. Squares: unmodified octyl-EV; triangles: metallic phosphate modified octyl-EV; circles: EV immobilized on metallic phosphate octyl-agarose.

3.4 Conclusion

The modification of immobilized enzymes with metallic phosphate enable to have some of the advantages achieved using the nanoflower production method to immobilize lipase. This method is a very simple to perform one and can be assayed with any immobilized enzyme, not only lipases. As it does not require the use of any special expertise or facility, can be performed

in a very short time by any researcher that requires the improvement of the immobilized enzyme features (even using commercial immobilized enzyme preparations). This effect is achieved if the enzyme is treated with the metal phosphate salts, the metal chloride or the phosphate alone have a negative effect on enzyme stability when present in the inactivation solution, and the immobilization on modified support has a smaller impact on enzyme stability (and in some instances is even negative, increasing the stabilization achieved by the modification of the immobilized enzyme). That way, we can hypothesize that this positive effect of the modification in some of the studied lipases is caused by the modification of the nucleation points of the protein structure, even if the nanoflower structures are not produced. This suggested that part of the benefices of the enzyme immobilization using nanoflowers maybe this modification of the enzyme groups and not only the global coating of the enzyme surface by the metal shell. This way, this can be of general applicability to other immobilized enzymes, with the advantage of the possibility of selecting the mechanical properties of the final biocatalyst. Ongoing research in our laboratory suggests a dependence of the immobilization protocol applied to the lipase and the effects of the treatment with metal phosphate.

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APPENDIX

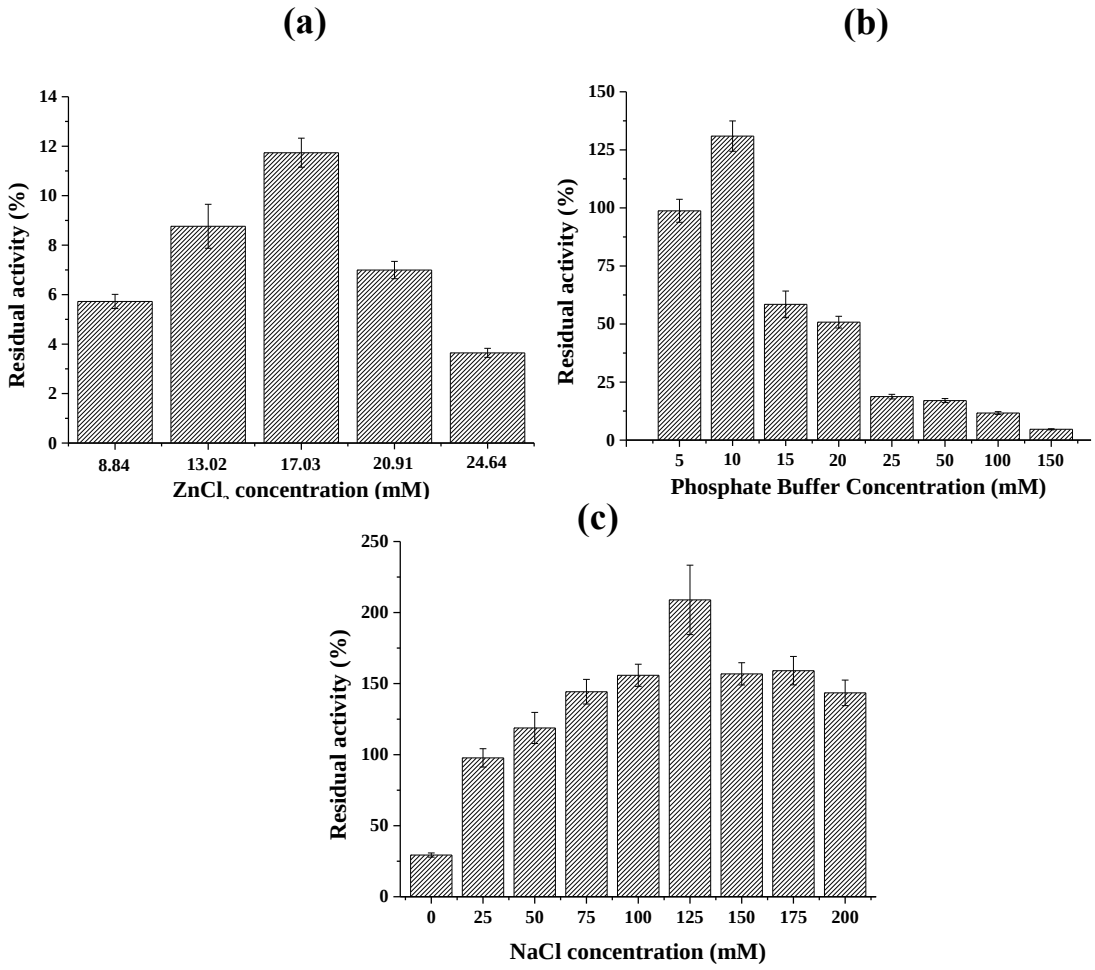

Figure A3.1. Effect of the concentration of $ZnCl_2$ **(a)**, phosphate buffer **(b)** and NaCl **(c)** on residual activity of ZnP(TLL)-NFs using a protein load of 0.05 mg/mL. The other conditions were unchanged. Experiments were performed as described in Methods.

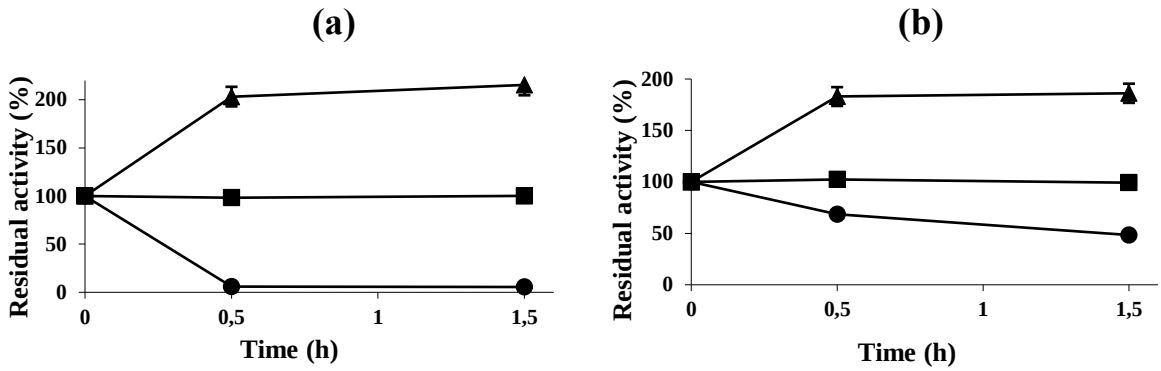

Figure A3.2. Immobilization courses of TLL on octyl agarose using enzyme loadings of **(a)** 1 mg/g and **(b)** 20 mg/g. The immobilization was performed in 5 mM sodium phosphate at 25 °C and pH 7.0. Squares: reference; triangles: suspension and circles: supernatant. Other specifications are described in Methods.

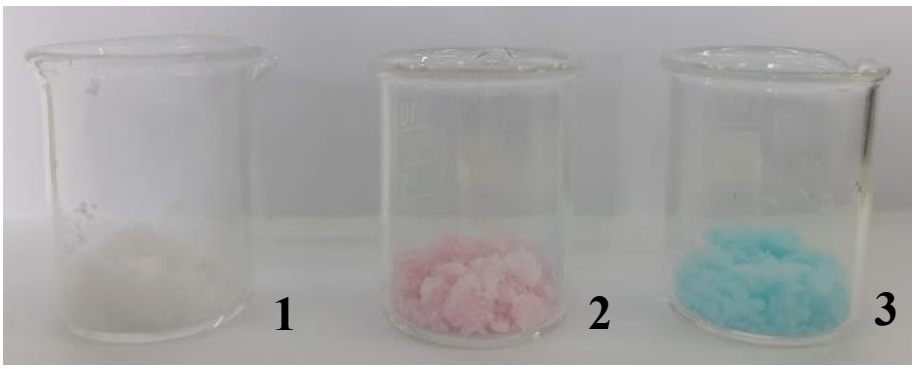

Figure A3.3. Octyl-TLL biocatalyst (1 mg/g) modified with (1) $ZnCl_2$/sodium phosphate, (2) $CoCl_2$/sodium phosphate and (3) $CuCl_2$/sodium phosphate. Other specifications are described in Methods.

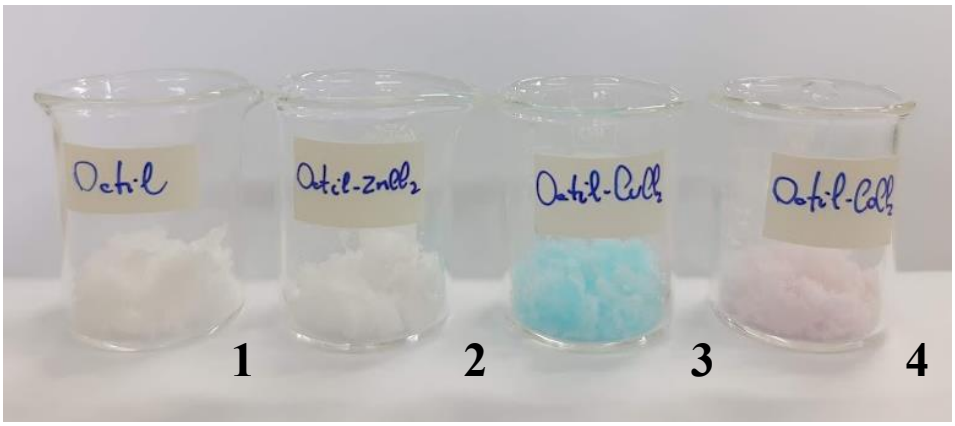

Figure A3.4. TLL (1 mg/g) immobilized on (1) octyl agarose and (2-4) octyl agarose modified with: (2) $ZnCl_2$/sodium phosphate, (3) $CuCl_2$/sodium phosphate and (4) $CoCl_2$/sodium

phosphate. The immobilization was performed in 5 mM sodium phosphate at 25 °C and pH 7.0. Other specifications are described in Methods.

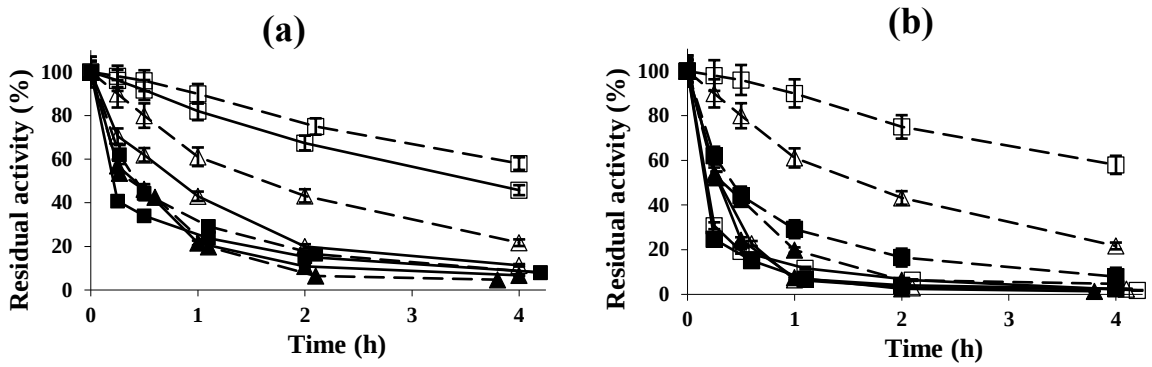

Figure A3.5. Effect of 17 mM $ZnCl_2$ (a) or 10 mM phosphate buffer (b) on the inactivations courses of different TLL-biocatalysts. The inactivation was performed at 75 °C and pH 7.0. Other specifications are described in Methods. The solid symbols correspond to unmodified octyl-TLL and the empty symbols to octyl-TLL modified with $ZnCl_2$/sodium phosphate. The biocatalysts loadings were: 1 mg/g (triangles) and 20 mg/g (squares). The dotted line corresponds to inactivation in 10 mM Tris buffer as a reference.

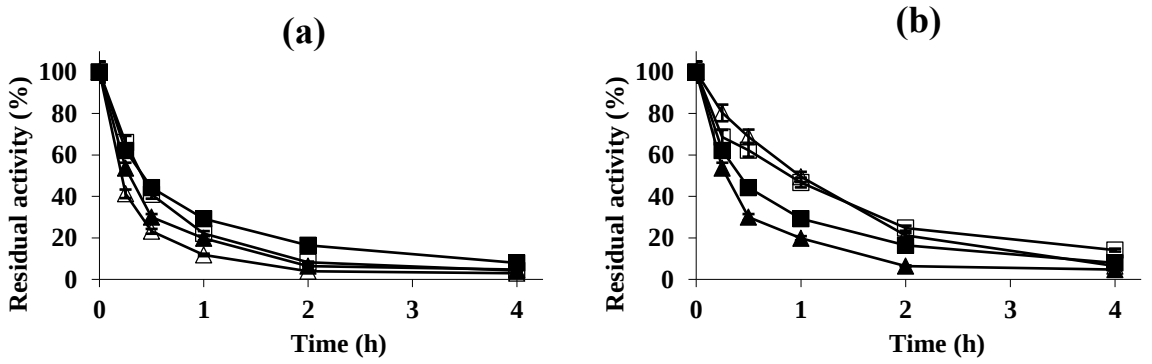

Figure A3.6. Effect of the modification of immobilized TLL with $CuCl_2$/sodium phosphate (a) and $CoCl_2$/sodium phosphate (b). The inactivation was performed in 10 mM Tris at pH 7.0 and 75 °C. Other specifications are described in Methods. The solid symbols correspond to unmodified octyl-TLL and the empty symbols the modified ones. The enzyme loadings are: 1 mg/g (triangles) and 20 mg/g (squares).

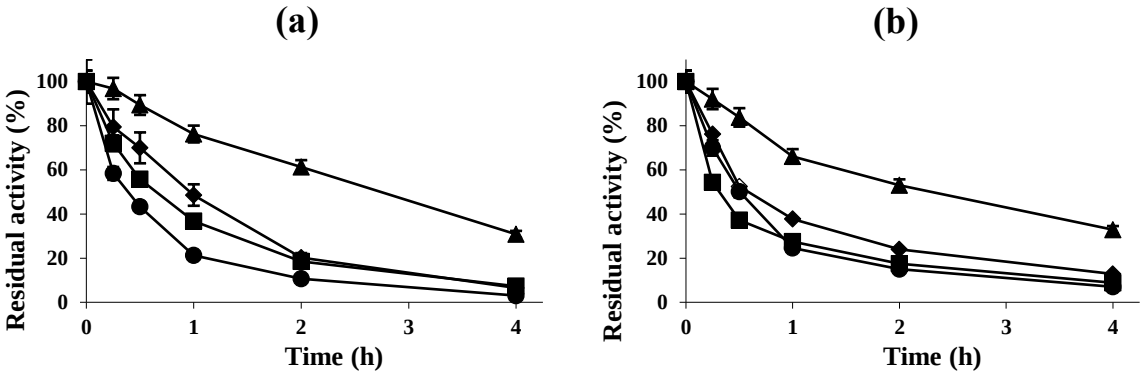

Figure A3.7. Inactivation courses of octyl-CoP-TLL further treated with with $ZnCl_2$/sodium phosphate and with $CuCl_2$/sodium phosphate for enzyme loadings of **(a)** 1 mg/g and **(b)** 20 mg/g. The inactivation was performed with 10 mM Tris buffer at pH 7.0 and 75 °C. Squares: Octyl-TLL; triangles: Octyl-CoP-TLL-ZnP; circles: Octyl-CoP-TLL-CuP., Rhombus: Octyl-CoP-TLL; Other specifications are described in Methods.

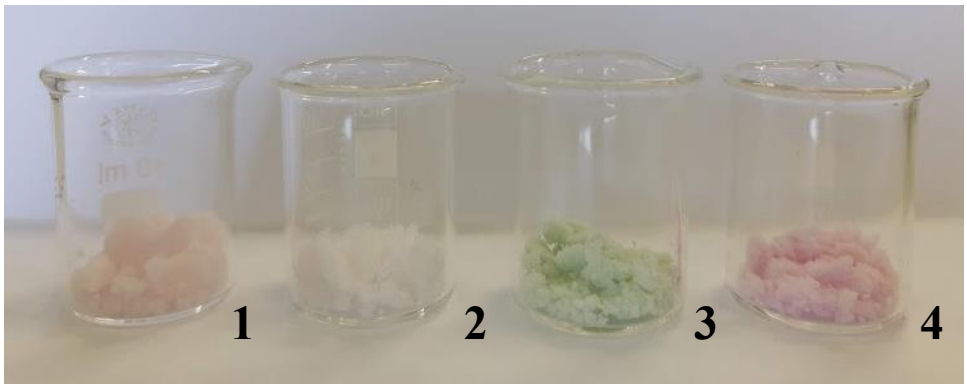

Figure A3.8. Octyl-CoP-TLL (1 mg/g) (1) and m after further modification with: $ZnCl_2$/sodium phosphate (2), $CuCl_2$/sodium phosphate (3) and $CoCl_2$/sodium phosphate (4). Other specifications are described in Methods.

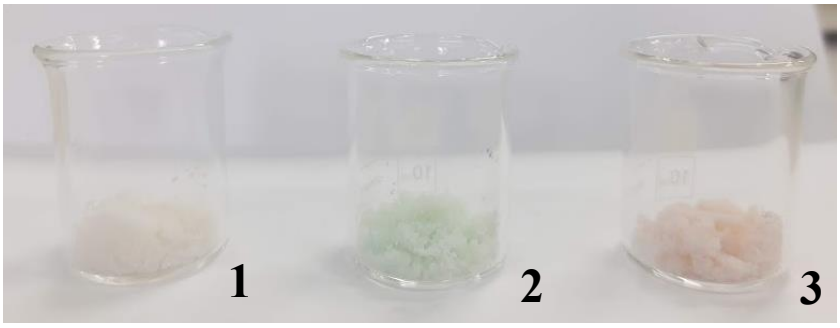

Figure A3.9. Purolite-TLL (20 mg/g) modified with (1) $ZnCl_2$/sodium phosphate, (2) $CuCl_2$/sodium phosphate and (3) $CoCl_2$/sodium phosphate. Other specifications are described in Methods.

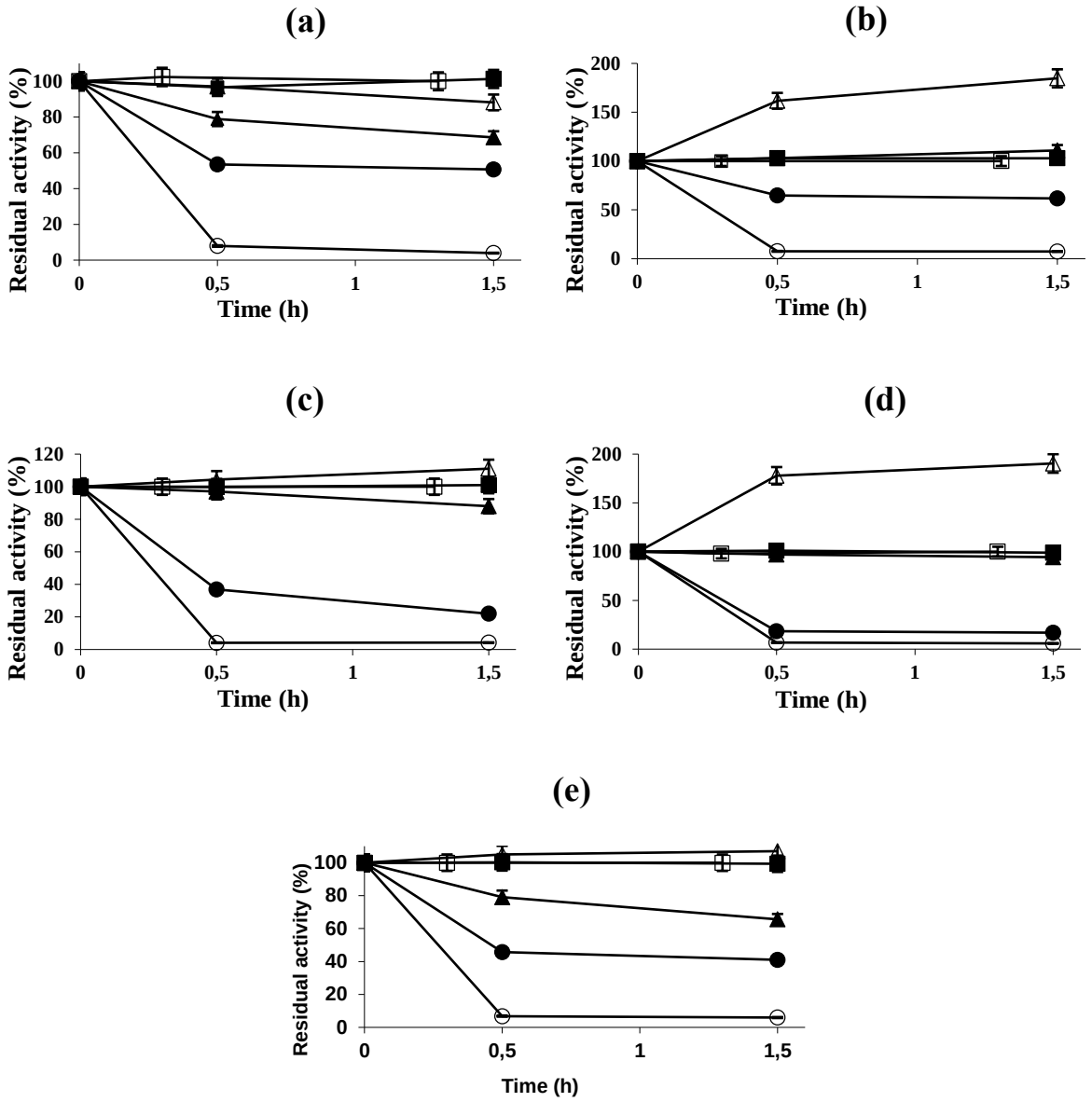

Figure A3.10. Immobilization courses of (a) CALB, (b) CALA, (c) CRL, (d) RML, and (e) EV on octyl agarose using enzyme loadings of 1 mg/g and 20 mg/g. The immobilization was performed in 5 mM sodium phosphate at 25 °C and pH 7.0. Solid symbols correspond to 1 mg/g enzyme loading and empty symbols to 20 mg/g. Solid squares: reference; solid triangles: suspension and solid circles: supernatant. Other specifications are described in Methods.

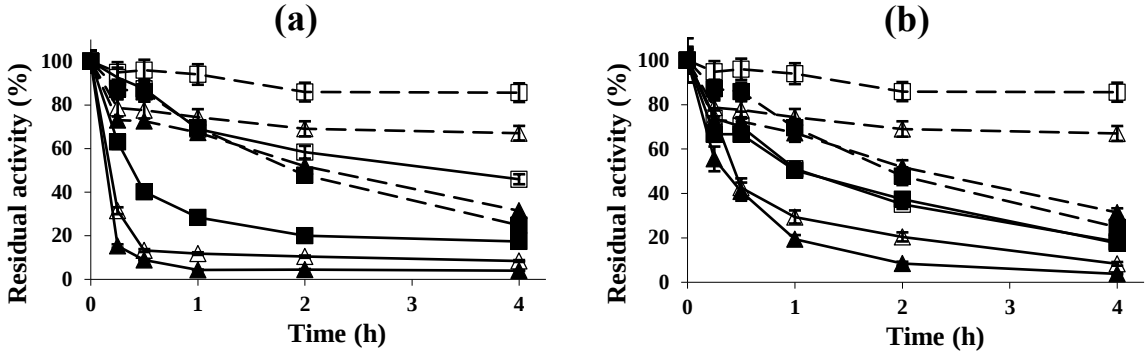

Figure A3.11. Effect of (a) 17 mM $ZnCl_2$ and (b) 10 mM sodium phosphate buffer on the inactivation courses of different octyl-CALA biocatalysts in 10 mM Tris buffer at pH 7.0 and 85°C. Other specifications are described in Methods. The solid symbols correspond to unmodified octyl-CALA and the empty symbols to modified octyl-CALA-ZnP. The enzyme loadings were: 1 mg/g (triangles) and 20 mg/g (squares). The dotted line corresponds to inactivation in 10 mM Tris buffer as a reference.

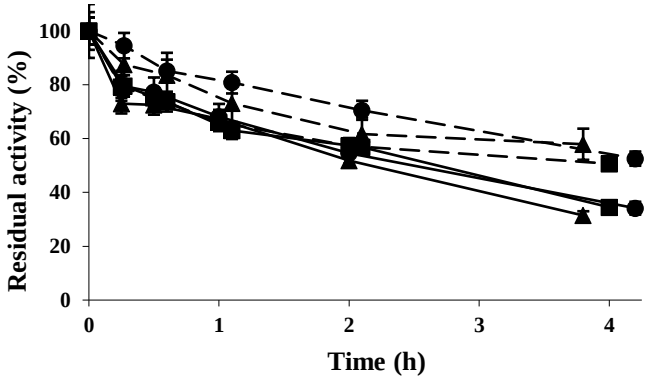

Figure A3.12. Inactivation course of untreated and treated octyl-CALA. The biocatalysts were inactivated in 10 mM Tris at pH 7 and 85 °C. Other specifications are described in Methods. The enzyme loadings are: 1 mg/g (solid line) and 20 mg/g (dotted line). Untreated biocatalysts (triangles); biocatalyst treated with phosphate (squares) and $ZnCl_2$ (circles).

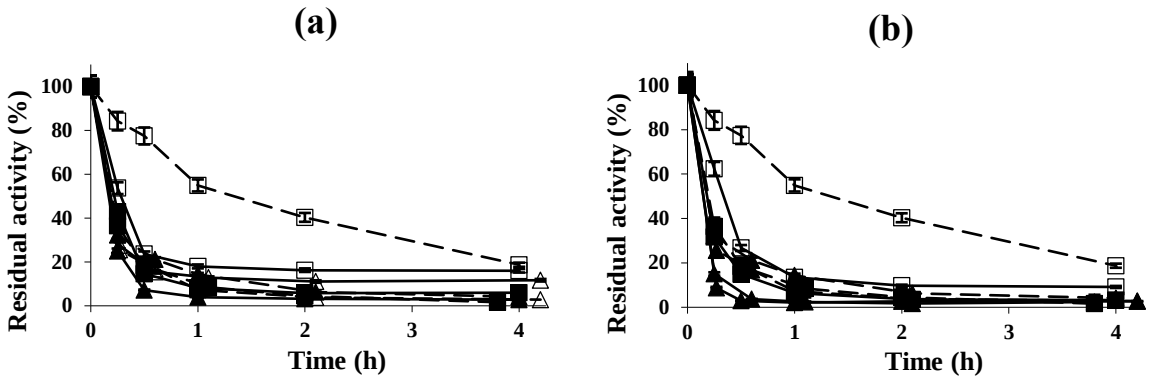

Figure A3.13. Effect of **(a)** 17 mM $ZnCl_2$ and **(b)** 10 mM sodium phosphate buffer in the inactivation courses of different octyl-CRL biocatalysts. The inactivation was performed at 70 °C and pH 7. Other specifications are described in Methods. The solid symbols correspond to unmodified octyl-CRL and the empty symbols to octyl-CRL-ZnP. The enzyme loadings are: 1 mg/g (triangles) and 30 mg/g (squares). The dotted line corresponds to inactivation in 10 mM Tris buffer as a reference.

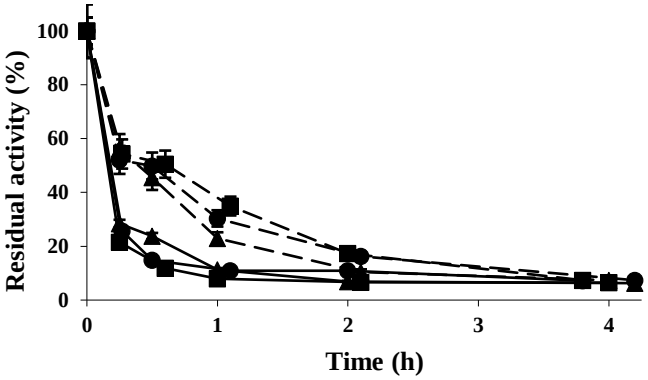

Figure A3.14. Inactivation course of untreated and treated octyl-CRL. The biocatalysts were inactivated in 10 mM Tris at pH 7 and 70 °C. Other specifications are described in Methods. The enzyme loadings are: 1 mg/g (solid line) and 30 mg/g (dotted line). Untreated biocatalysts (triangles); biocatalyst treated with phosphate (squares) and $ZnCl_2$ (circles).

Table A3.1. Hydrolytic activities of TLL immobilized on octyl agarose modified with $CoCl_2$/sodium phosphate and subsequently treated with different metallic phosphates. The hydrolytic activities were measured using 1 mM *p*-NPB at pH 7.0 and 25 °C. Experiments were conducted as described in the Methods.

Biocatalysts	**Activity with *p*-NPB (U/g)**	
	1 mg/g	**20 mg/g**
Octyl-CoP-TLL	94.33 ± 3.51	176.09 ± 11.87
Octyl-CoP-TLL-CoP	111.37 ± 5.85	205.63 ± 13.70
Octyl-CoP-TLL-CuP	86.19 ± 5.20	213.05 ± 11.57
Octyl-CoP-TLL-ZnP	107.05 ± 4.75	190.58 ± 7.67

Table A3.2. Effect of the treatment with phosphate and chloride metallic salt on the specific activity of lowly and highly loaded octyl-CALA biocatalysts. The hydrolytic activity was measured employing 1 mM *p*-NPB at pH 7.0 and 25 °C. Experiments were performed as described in Methods.

Offered Enzyme	**Activity with *p*-NPB (U/g)**		
	Treatment		
	No treatament	**Phosphate**	**$ZnCl_2$**
1 mg/g	103.09 ± 4.17	110.07 ± 4.51	100.27 ± 3.013
20 mg/g	158.12 ± 7.91	159.13 ± 5.95	155.39 ± 6.77

Table A3.3. Effect of the treatment with phosphate and chloride metallic salt on the specific activity of lowly and highly loaded octyl-CRL biocatalysts. The hydrolytic activity was measured employing 1 mM *p*-NPB at pH 7.0 and 25 °C. Experiments were performed as described in Methods.

Offered Enzyme	**Activity with *p*-NPB (U/g)**		
	Treatment		
	No treatament	**Phosphate**	**$ZnCl_2$**
1 mg/g	119.77 ± 3.99	110.07 ± 4.51	123.47 ± 5.17
20 mg/g	168.22 ± 7.41	173.92 ± 7.69	179.43 ± 8.97

CHAPTER 4
TUNING IMMOBILIZED COMMERCIAL LIPASE PREPARATIONS FEATURES BY SIMPLE TREATMENT WITH METALLIC PHOSPHATE SALTS

Four commercial immobilized lipases biocatalysts have been submitted to modifications with different metal (zinc, cobalt, or copper) phosphates to check the effects of this modification on enzyme features. The lipase preparations were Lipozyme®TL (TLL-IM, lipase from *Thermomyces lanuginosus*), Lipozyme®435 (L435, lipase B from *Candida antarctica*), Lipozyme®RM (RML-IM), and LipuraSelect (LS-IM), both from lipase from *Rhizomucor miehei***.** The modifications greatly altered enzyme specificity, increasing the activity versus some substrates (e.g., TLL-IM modified with zinc phosphate in hydrolysis of triacetin) while decreasing the activity versus other substrates (the same preparation in activity versus *R* or *S* methyl mandelate). Enantiospecificity was also drastically altered after these modifications, e.g., LS-IM increased the activity versus the *R* isomer while decreasing the activity versus the *S* isomer when treated with copper phosphate. Regarding the enzyme stability, it was significantly improved using octyl-agarose-lipases. Using all these commercial biocatalysts no significant positive effects were found, in fact a decrease in enzyme stability was usually detected. The results point towards the possibility of a battery of biocatalysts including many different metal phosphates and immobilization protocols being a good opportunity to tune enzyme features, increasing the possibilities of having biocatalysts that may be suitable for a specific process.

4.1 Introduction

Lipases are among the most utilized enzymes, both academically and industrially [1–5]. This is because they are very robust biocatalysts, able to perform a wide variety of reactions such as hydrolysis, esterifications [6–9], transesterifications [10–14] or acidolysis [15,16]. They can be used in a wide variety of reaction media (aqueous medium, organic solvents [17,18], solvent-free [19], ionic liquid [20–22], deep eutectic solvents [23], and supercritical fluids [24–27]) and have a wide substrate specificity, accepting substrates that are very different structurally. However, this is in many instances bound to a high regioselectivity, enantiospecificity and selectivity [28–34].

In fact, they are one of the most successful examples of enzymes presenting promiscuous activities [1,35–40].

In homogeneous media, most lipases present a conformational equilibrium between a form where a polypeptide chain (lid) isolates its active center from the medium (closed form) and a form where this lid is shifted exposing the active center to the medium (open form) [41–43]. In the presence of insoluble drops of substrate, the open form of the lipase becomes adsorbed on the hydrophobic surface and becomes stabilized [42]. This can occur also on any other hydrophobic surface such as the open form of other lipase molecule [44–48], a hydrophobic protein [49,50] or a hydrophobic matrix [51].

The use of immobilized enzymes permits enzyme recovery and their reuse, providing that the enzyme remains active [52–59]. This also enables a simpler control of the reaction and also the utilization of any reactor configuration [60]. Researchers have tried to couple this immobilization step in the design of industrial enzyme biocatalysts in a way to improve many enzyme features. That way, a proper immobilization may improve enzyme stability by different reasons (recently reviewed [61]), and that way increase the range of conditions where the enzyme may be utilized, increasing the prospect of success in the design of a bioprocess [62,63]. Furthermore, enzyme immobilization will alter enzyme selectivity, specificity and activity [28], may reduce inhibitions and, if adequately designed, enable the one step immobilization-purification of the target enzyme [64–66].

In the last decade, the production of hybrid enzyme nanoflowers proved to be an immobilization method able to improve some enzyme features, such as enzyme stability and activity [67–74]. In some cases, this technique has been applied to immobilize lipases [75–88].

However, the mechanical fragility of nanoflowers makes their use complex in most reactor configurations. One alternative to solve this problem in some instances is to trap the nanoflowers in solids with better mechanical performance or confer a magnetic character to the biocatalyst [67,76,77,85,89–91].

Recently, we tried to reproduce this strategy using immobilized enzymes [92]. We found that in some instances the enzyme features (activity or stability) were significantly improved [92]. Although there was no evidence of the production of hybrid enzyme-lipase nanoflower structures, the modification of the enzyme nucleation sites with the metal phosphate was assumed to be the cause of these positive effects.

In this new research, we investigate if this simple immobilized enzyme treatment may improve some of the most utilized commercial immobilized lipase preparations. The lipase B from *Candida antarctica* immobilized on moderately hydrophobic Lewatit VP OC 1600 via

interfacial activation [93], with commercial name Novozym® 435 [93], has been one of the used preparations. Also, the lipase from *Rhizomucor miehei* immobilized on Duolite ES 562, a weak anion-exchange resin based on phenol-formaldehyde copolymers (RML-IM) has been included in this study [94] and a new biocatalyst called LipuraSelect, with scarce information available on the preparation way (that is, the immobilization mechanism is unknown). Finally, the lipase from *Thermomyces lanuginosus* immobilized on a cationic silicate (TLL-IM) was included [95]. All these commercial preparations have been treated with phosphate and the chloride salts of Cu^{2+}, Co^{2+} and Zn^{2+}, and their functional properties have been analyzed.

4.2 Materials and Methods

4.2.1 Materials

In this study, we have employed different commercial immobilized lipase. Lipozyme®TL (TLL-IM), Lipozyme®435 (L435), Lipozyme®RM (RML-IM), and LipuraSelect (LS-IM) were kindly donated by Novozymes Spain (Madrid, Spain). Triacetin, (*R*)- and (*S*)-methyl mandelate, zinc chloride ($ZnCl_2$), copper chloride ($CuCl_2$), cobalt chloride ($CoCl_2$), sodium chloride (NaCl) and acetonitrile for HPLC (gradient grade, ≥99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

4.2.2 Methods

4.2.2.1 Modification of immobilized enzyme with metallic salt/phosphate

TLL-IM, L435, RML-IM, and LS-IM were modified with metallic salt/phosphate following the procedure described by Guimarães et al. [92]. A mass of 1 g of immobilized enzyme was suspended in 10 mL of saline buffer (10 mM sodium phosphate buffer and 125 mM NaCl) at pH 7.4 and, then, 400 µL of 230 mM of the corresponding metal salt was added. The enzyme treatment was conducted at room temperature under gentle stirring for 5 h in an orbital shaker at 550 rpm. After modification, the suspension was filtered and the biocatalysts were washed with distilled water (10 times with 10 volumes of water), and stored at 4 °C.

4.2.2.2 Thermal inactivations of the different lipase preparations

In a standard experiment, 1 g of immobilized biocatalyst was suspended in 10 mL of 10 mM Tris-hydrochloride (Tris-HCL) at pH 7.0 and incubated at different temperatures. Phosphate was discarded as medium to inactivate the immobilized enzymes as it has been

reported to be negative for enzyme stability. The low buffer concentration prevents risks of enzyme release for the biocatalysts based on ion exchange [96,97].

The temperatures were selected to have reliable but not too long half- lives of the unmodified immobilized enzymes (TLL-IM and L435: 75 °C; RML-IM and LS-IM: 60 °C). Periodically, samples of 0.3 mL of inactivation suspension were collected after homogenization using a pipette with cut tip to determine their residual activities. Residual activities were defined as activity of the biocatalyst after the indicated inactivation time divided by its initial and expressed in percentage. The experiments were performed employing triacetin as substrate for immobilized biocatalyst.

4.2.2.3 Enzyme activity assays

One unit of activity (U) was defined as the amount of enzyme that hydrolyzes one µmol of substrate per minute under the described conditions.

4.2.2.3.1 Hydrolysis of triacetin

A volume of 0.3 mL of immobilized enzyme suspension (166 mg/mL) was added to 3 mL of 50 mM of triacetin prepared in 50 mM of sodium phosphate buffer at pH 7.0. Hydrolysis was carried out at 25 °C under magnetic stirring (100 rpm). The hydrolytic activity in triacetin was quantified by detection of 1,2 and 1,3 diacetin (under these conditions 1,2 diacetin suffer acyl migration giving 1,3 diacetin) [98]. The degree of conversion was calculated by HPLC in a Waters 486 chromatograph (Waters, Millford, USA) equipped with a UV/VIS detector (set to 230 nm) [98] using a Kromasil C18 column (15 cm x 0.46 cm) with a mobile phase composed of 85% (v/v) of water and 15% (v/v) of acetonitrile with a flow rate of 1 mL/min. The retention times were 4 min for 1,2 and 1,3 diacetins (under these conditions eluted at the same time) and 18 min for triacetin. Conversions between 15-20% were used to calculate enzyme activity [99].

4.2.2.3.2 Hydrolysis of R- or S-methyl mandelate

A mass of 0.05 g of commercial immobilized lipase were added to 3 mL of 50 mM *R*- or *S*-methyl mandelate in 50 mM sodium phosphate buffer solution at pH 7.0. Hydrolysis was carried out at 25 °C under magnetic stirring (100 rpm). The substrate and product concentrations were determined by HPLC using a Waters 486 chromatograph (Waters, Millford, USA) equipped with a UV/VIS detector (set to 230 nm) [100] using a Kromasil C18 column (15 cm x 0.46 cm) with a mobile phase composed 10 mM ammonium acetate and acetonitrile (65-35% (v/v)) at pH 2.8 with a flow rate of 1 mL/min. The retention times were 2.5 min for mandelic

acid and 4.2 min for the *R*- or *S*-methyl mandelate. Conversions between 15-25% were used to calculate enzyme activity [101].

4.3 Results and Discussion

4.3.1 Modification of commercial immobilized TLL (TLL-IM)

TLL-IM was modified as indicated in methods and the activities of the different biocatalysts versus diverse substrates were determined (Table 4.1). The modification with phosphate and Co^{2+} produced a significant decrease of the activity of TLL-IM versus triacetin, almost by 50%. However, the activity significantly increased using Cu^{2+} (by a 40%) and even more significantly using Zn^{2+} (almost a 70%). However, all treatments produced a decrease in the activity versus both methyl mandelate isomers, more significant for the S-isomer. This means that the treatment alters both specificity versus the substrates and enantiospecificity. In the most significant cases, the activity ratio of activities between *R*-methyl mandelate and triacetin (that is initially just under 1.3) increases to 2 when the enzyme is modified using phosphate and Co^{2+}, or to 0.6 using phosphate and Zn^{2+}. The enantiospecificity for the isomers of methyl mandelate also changes, but not so significantly from the initial ratio of reaction rate using *R*/*S* isomer of almost 1.6, to almost 2 if the biocatalyst is modified with phosphate and Co^{2+}. These changes suggested that the metal phosphate modification should produce quite large changes in the functional properties of the enzyme (very likely caused by enzyme conformational changes) as reported in many papers regarding the effects of the immobilization protocol [102–111] or the chemical or physical modification of the enzymes [112–118] on enzyme specificity or selectivity. The fact that an enzyme is immobilized did not mean that the enzyme mobility is fully suppressed, and chemical or physical modifications can induce conformational changes. For example, it has been showed that the blocking of TLL-octyl-vinyl sulfone biocatalyst with different reagents can fully alter the enzyme functionality and also the enzyme structure [100].

Table 4.1. Specific activity of different biocatalysts with 50 mM *R*- or *S*-methyl mandelate (pH 7, 25 °C), 50 mM of triacetin (pH 7, 25 °C). Experiments were performed as described in Methods.

Biocatalysts	**Activity (U/g)**		
	Triacetin	***R*-Mandelate**	***S*-Mandelate**
TLL-IM	7.73 ± 0.35	9.90 ± 0.49	6.33 ± 0.57
TLL-IM-ZnP	13.02 ± 0.64	7.88 ± 0.27	5.31 ± 0.18
TLL-IM-CuP	10.80 ± 0.44	8.77 ± 0.37	5.12 ± 0.26
TLL-IM-CoP	4.08 ± 0.19	8.09 ± 0.40	4.18 ± 0.32

Figure 4.1 shows the inactivation course of the different TLL-IM biocatalysts. While Co^{2+}/Cu^{2+} and phosphate treatment produced a drastic decrease in enzyme stability, the treatment with Zn^{2+}resulted in a biocatalyst that fully maintained the enzyme stability at pH 7. These results disagree with the results obtained using octyl-agarose-TLL [92], where stability was greatly improved after this treatment, while the activity (versus *p*-NPB) was slightly decreased. This suggested that the immobilization protocol could greatly alter the effect of the metal phosphate modification of the biocatalyst. This result agrees with previous reports that state that the immobilization protocol can alter the effect of the enzyme modification on enzyme features, either chemical or physical [117,118]. In the case of TLL-IM, the modification with phosphate and Zn^{2+} permitted to increase the enzyme activity versus some substrates, altering enzyme specificity, while maintaining enzyme stability.

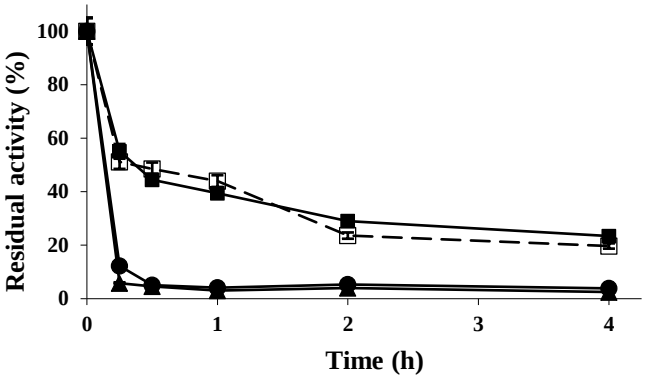

Figure 4.1. Inactivation courses of TLL-IM unmodified and modified with metallic salt/sodium phosphate. The inactivation was performed with 10 mM Tris buffer at pH 7.0 and 75 °C. Other specifications are described in Methods. Unmodified TLL-IM (open squares and dotted line);

TLL-IM modified with $ZnCl_2$/sodium phosphate (solid squares); $CuCl_2$/sodium phosphate (solid circles); $CoCl_2$/sodium phosphate (solid triangles).

4.3.2 Modification of commercial immobilized CALB (L435)

Table 4.2 shows the effect of the modification of L435 with different metal phosphate on the enzyme activities versus different substrates. Using triacetin, Zn^{2+} and phosphate treatment produced a 25% increase on enzyme activity, while the other two salts have a marginal negative effect. Using both isomers of methyl mandelate, the decrease in activity was more significant, to 1/3 using phosphate and Co^{2+} for the *R*-isomer, and 1/5 using the *R*-isomer. This produced a great effect on enzyme specificity, while the activity versus triacetin/ activity versus *R*-isomer of the initial biocatalyst was 2.9 for the unmodified enzyme, this increased to 6.5 for the enzyme modified using Zn^{2+} and phosphate, 5.9 using Cu^{2+} or 8.5 using Co^{2+}. Regarding the activity versus *R*/activity versus *S*-isomers, they in the range of 1.3-1.4 for the initial preparation or the biocatalysts treated with zinc phosphate, 1.9 if treated with copper phosphate, and over 2 if treated using cobalt phosphate.

Table 4.2. Specific activity of different biocatalysts with 50 mM *R*- or *S*-methyl mandelate (pH 7, 25 °C), 50 mM of triacetin (pH 7, 25 °C). Experiments were performed as described in Methods.

Biocatalysts	**Activity (U/g)**		
	Triacetin	***R*-Mandelate**	***S*-Mandelate**
L435	119.0 ± 5.2	42.6 ± 1.8	31.2 ± 1.6
L435-ZnP	149.9 ± 7.8	23.1 ± 0.9	17.3 ± 0.9
L435-CuP	113.9 ± 5.9	19.4 ± 1.0	11.5 ± 0.6
L435-CoP	116.2 ± 6.9	13.3 ± 0.6	6.4 ± 0.4

Figure 4.2 shows the inactivation courses, and it becomes obvious that all the modifications presented a similarly small negative effect on enzyme stability at pH 7. Using octyl-agarose-CALB, the results were again quite different, with some increase on enzyme stability and activity. Explanations for these results may be similar to those given in the case of TLL.

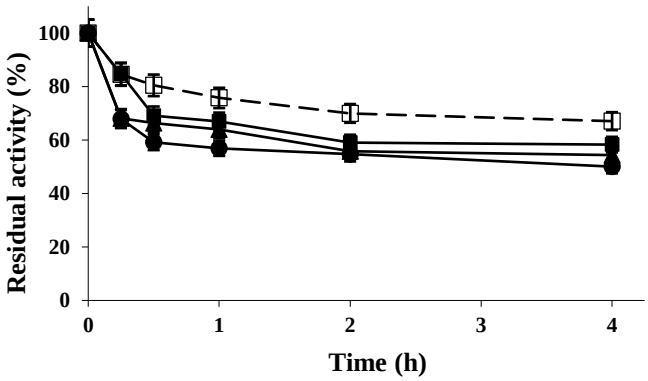

Figure 4.2. Inactivation courses of L435 unmodified and modified with metallic salt/sodium phosphate. The inactivation was performed with 10 mM Tris buffer at pH 7.0 and 75 °C. Other specifications are described in Methods. Unmodified L435 (open squares and dotted line); L435 modified with $ZnCl_2$/sodium phosphate (solid squares); $CuCl_2$/sodium phosphate (solid circles); $CoCl_2$/sodium phosphate (solid triangles).

4.3.3 Modification of commercial immobilized RML (RM-IM and LS-IM)

In the case of RML, we have got two different commercial preparations, RML-IM and LS-IM. The activities of both biocatalysts (intact and metal phosphate modified) may be found on Tables 4.3 (RML-IM) and 4.4 (LS-IM). First, we will compare the activities of both biocatalysts versus the different substrates used in this study. RML-IM was slightly more active versus triacetin than LS-IM, however it was significantly more active (almost by a 10-fold factor) versus *R*-methyl mandelate. The hydrolysis of the *R*-isomer was more rapid using RML-IM (1.15-fold) than using LS-IM, while LS-IM preferred the *S*-isomer (1.4-fold). That way, both RML biocatalysts presented a very different specificity and enantiospecificity, as it has been reported in many other instances for RML immobilized on different supports [102–107].

When RML-IM was treated with the metal salts, the activity versus triacetin decreased much more than the activity versus the methyl mandelate esters. The activity versus triacetin decreased to 81% when the biocatalyst was modified with zinc or cobalt phosphates, but below 50% if using copper phosphate. The activity versus *R*-methyl mandelate decreased to around 10% in the more drastic case (when modified with zinc phosphate), while using the *S*-isomer the activity was maintained. That way, the activity versus triacetin/activity versus *R*-methyl mandelate ratio was moved from 7.6 for the original biocatalyst, to 3.7 for the enzyme modified

with copper phosphate. The changes in the *R*/*S* methyl mandelate activity ratio were much smaller (from the original 1.15 to almost 1).

Table 4.3. Specific activity of different biocatalysts with 50 mM *R*- or *S*-methyl mandelate (pH 7, 25 °C), 50 mM of triacetin (pH 7, 25 °C). Experiments were performed as described in Methods.

Biocatalysts	**Activity (U/g)**		
	Triacetin	***R*-Mandelate**	***S*-Mandelate**
RML-IM	86.2 ± 4.7	11.3 ± 0.8	9.8 ± 0.5
RML-IM-ZnP	70.00 ± 3.7	9.9 ± 0.6	9.8 ± 0.4
RML-IM-CuP	38.4 ± 1.9	10.2 ± 0.6	9.8 ± 0.4
RML-IM-CoP	69.9 ± 3.8	10.1 ± 0.5	9.9 ± 0.2

Table 4.4. Specific activity of different biocatalysts with 50 mM *R*- or *S*-methyl mandelate (pH 7, 25 °C), 50 mM of triacetin (pH 7, 25 °C). Experiments were performed as described in Methods.

Biocatalysts	**Activity (U/g)**		
	Triacetin	***R*-Mandelate**	***S*-Mandelate**
LS-IM	80.5 ± 4.9	0.92 ± 0.04	1.29 ± 0.06
LS-IM-ZnP	54.9 ± 3.0	0.73 ± 0.04	0.67 ± 0.03
LS-IM-CuP	63.8 ± 3.3	1.50 ± 0.09	0.88 ± 0.06
LS-IM-CoP	63.7 ± 3.9	1.10 ± 0.07	0.99 ± 0.05

Results were very different using LS-IM. Activity versus triacetin decreased after the treatment, but it decreased more using zinc (below 70%), while the other two preparations maintained around 80% of the initial activity. In the case of methyl mandelate, the effects were very different using each of the isomers. The activity versus *R*-methyl mandelate increased by 60% when treated with copper, by 20% using cobalt, while the use of zinc salts decreased the activity by 20%. However, the activity versus the *R*-isomer decreased in all cases, more using zinc (to 50%) and less using cobalt (75%). This means some changes in enzyme specificity,

going from an activity of 62 versus triacetin/*S* methyl mandelate (much higher than using RM-IM) to 82 (after modification with Zn), and more significant changes in the activity versus *S*/*R* methyl mandelate ratio. The unmodified biocatalyst presented an activity ratio of 1.4. All modified biocatalysts preferred the *R*-isomer, giving a value around 0.6 when modified with copper. That way for this preparation the modification produced a more significant change in the enantiospecificity than in the specificity, in opposition with RML-IM.

The comparison between two biocatalysts of the same enzyme confirms that the effect of the modification with metal phosphate is greatly dependent on the immobilization protocol, such as it had been reported for other physical or chemical immobilized enzyme modifications. [112,115–119].

Figure 4.3 shows the effect of the metal phosphate treatments on the stability of RML-IM. All the treatments had a scarce, but positive effect on enzyme stability. The comparison with Figure 4.4 shows that LS-IM is much more stable than RML-IM. The modification of LS-IM with zinc phosphate had no effect on enzyme stability while the other two treatments produced a clear decrease on enzyme stability. Again, different enzyme preparations of the same enzyme exhibited a very different response to the treatment with metal phosphate.

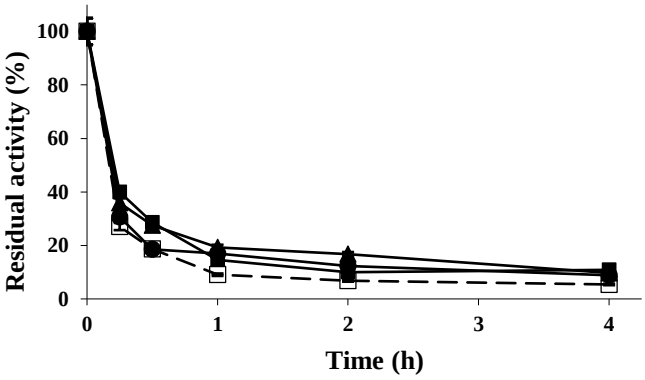

Figure 4.3. Inactivation courses of RML-IM unmodified and modified with metallic salt/sodium phosphate. The inactivation was performed with 10 mM Tris buffer at pH 7.0 and 60 °C. Other specifications are described in Methods. Unmodified RML-IM (open squares and dotted line); RML-IM modified with $ZnCl_2$/sodium phosphate (solid squares); $CuCl_2$/sodium phosphate (solid circles); $CoCl_2$/sodium phosphate (solid triangles).

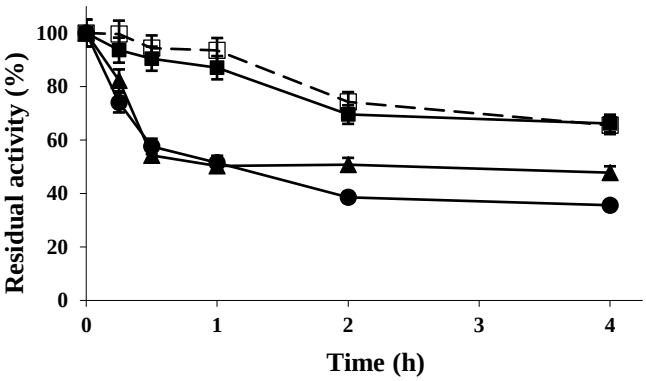

Figure 4.4. Inactivation courses of LS-IM unmodified and modified with metallic salt/sodium phosphate. The inactivation was performed with 10 mM Tris buffer at pH 7.0 and 60 °C. Other specifications are described in Methods. Unmodified LS-IM (open squares and dotted line); LS-IM modified with $ZnCl_2$/sodium phosphate (solid squares); $CuCl_2$/sodium phosphate (solid circles); $CoCl_2$/sodium phosphate (solid triangles).

4.4 Conclusions

The modification of commercial preparations of immobilized lipases with metal phosphate, in a treatment similar to the production of nanoflowers, alters enzyme specificity. These changes means that while the enzyme activity may increase for some substrates, it may decrease for some others. This also fully alters enzyme enantiospecificity. The best treatment is different for each enzyme, substrate, and even for each immobilization protocol. The results in this paper point that the immobilization protocol can play a critical role on the effects of the treatment. Regarding the stability, a feature that was reported as much improved using octyl-agarose-lipase, using the commercial preparations show a moderate impact, usually even decreasing enzyme stability, or producing marginal stabilization (in the case of RML-IM). Although in this paper we have focused on the effects of the modification on the functional properties of the immobilized enzymes, it seems very interesting to make deeper research in the mechanism of this modification. The fact that the metal crystals also growth on the naked supports makes this a very complex goal.

From the results of this paper, it is proposed that the modification of a biocatalyst with a battery of metals (not reducing the study to the ones used in this paper) may open new

opportunities for tailoring the enzyme features, increasing the opportunities to find a biocatalyst with the optimal properties for a specific process.

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CHAPTER 5
MINERALIZATION OF LIPASE FROM *Thermomyces lanuginosus* IMMOBILIZED ON METHACRYLATE BEADS BEARING OCTADECYL GROUPS TO IMPROVE ENZYME FEATURES

Lipase from *Thermomyces lanuginosus* (TLL) has been immobilized on Purolite Lifetech® ECR8806F (viz. methacrylate macroporous resin containing octadecyl groups, designated as Purolite C18-TLL) and the enzyme performance has been compared to that of the enzyme immobilized on octyl-agarose, designated as agarose C8-TLL. The hydrolytic activity versus *p*-nitrophenol butyrate decreased significantly, and to a lower extent versus *S*-methyl mandelate (more than twofold) while versus triacetin and *R*-methyl mandelate, the enzyme activity was higher for the biocatalyst prepared using Purolite C18 (up to almost 5 folds). Regarding the enzyme stability, Purolite C18-TLL was significantly more stable than the agarose C8-TLL. Next, the biocatalysts were mineralized using zinc, copper, or cobalt phosphates. Mineralization increased the hydrolytic activity of Purolite C18-TLL versus triacetin and *R*-methyl mandelate, while this activity decreased very significantly versus the *S*-isomer, while the effects using agarose C8-TLL was more diverse (hydrolytic activity increase or decrease was dependent on the metal and substrate). The zinc salt treatment increased the stability of both biocatalysts, but with a lower impact for Purolite C18-TLL than for agarose-C8-TLL. On the contrary, the copper and cobalt salts treatments decreased enzyme stability, but more intensively using Purolite C18-TLL. The results show that even using enzymes immobilized following the same strategy, the differences in the enzyme conformation causes the mineralization to have diverse effects on enzyme stability, hydrolytic activity, and specificity.

5.1 Introduction

The lipase from *Thermomyces lanuginosus* (TLL) is among the most utilized lipases worldwide due to its high stability and possibility of using it as catalyst of many different reactions [1]. The immobilization of this enzyme has shown to be a way to further improve the enzyme features [1].

Enzyme immobilization protocols can be designed to be coupled to enzyme purification [2], and enzyme stability may be improved by different paths (e.g., multipoint

covalent attachment, generation of favorable environments, etc.) [3]. Furthermore, enzyme inhibition, selectivity and specificity may be tuned in a quite random way through enzyme immobilization. This makes to have a wide library of biocatalysts of the same enzyme highly recommendable to have one biocatalyst with suitable features for a specific process [4]. That makes enzyme immobilization a critical point nowadays in the development of a biocatalyst [5–10] although to take full advantages of the technique, many aspects need to be considered, and some points require further research to have a full control of the process [11]. All these benefits may be also achieved when immobilizing TLL.

Immobilization of enzymes may be achieved using very diverse strategies. They can be immobilized on preexisting supports, with the advantages that this can have: the physical properties of the final biocatalysts will not depend on the enzyme, it is possible to select the mechanical properties of the biocatalyst as a function of the reactor, and it is possible to control the activation degree in the support without affecting the final mechanical properties of the biocatalyst, etc. [9]. They have also some problems [11]. The most obvious is the necessity of finding a support supplier and the costs of the support (including acquisition, storage, activation and discarding costs). Moreover, the enzyme will interact with only a percentage of its surface with the support (except using supports activated with polymers, or if the enzyme is later traded with a polymer, (e.g., polyethyleneimine) [12]. The other alternative is the immobilization forming ex novo supports. Examples of this strategy are the production of enzyme co-polymer [13,14], trapping of the enzymes (e.g., in alginate beads [15–17] or sol-gels [18–20]), cross-linked enzyme crystals [21–23], cross-linked enzyme aggregates [24–27], crystals coated with proteins [28–31], or nano-flowers [32–34]). These methods do not require a support but require other compounds that can be in certain cases relatively expensive (e.g., protein or polymer feeders). One advantage that is common to all of them is that the entire enzyme surface may be involved in the immobilization, that way, in general they can be very useful to stabilize complex multimeric enzymes where the first inactivation step was the subunit dissociation [35]. In some instances, like in the case of metal-organic framework (MOF), the solid may be prepared and then immobilize the enzyme (that is, using a pre-existing solid), or may be prepared in the presence of the enzyme forming the solid while the enzyme is immobilized at the same time (that is, forming an *ex novo* solid) [36–39].

Nanoflowers of lipases have been prepared in many instances and, in certain cases, this enzyme mineralization promotes improvements in its catalytic activity and/or stability [40-49]. However, their small size and mechanical fragility makes these biocatalysts not easy to be implemented in industry. This has been solved by using magnetic materials to facilitate their

handling [52–58] or by trapping the nanoflowers in larger and more resistant materials [59,60]. In this sense, recently, it has been proposed that the mineralization of previously immobilized enzymes can couple the advantages of enzyme mineralization and enzyme immobilization on preexisting supports, although the formation of proper nanoflowers cannot be observed [61,62]. These previous studies also showed that the enzyme conformation can play a critical role on the final effects of the mineralization [63,64]. This effect of protein conformation on the enzyme mineralization has been previously indicated by other authors using free enzymes [65], very recently another paper has shown that the crystals formed using a protein and 2-methylimidazole and zinc acetate metal–organic frameworks strongly depended on the protein structure and its molecular modifications [66].

As a general method of lipases immobilization (and of TLL in particular), the immobilization of the enzyme via interfacial activation on hydrophobic supports is advantageous, as it permits to have the open and monomeric form of the lipases in one step, improving its stability and purifying the lipases [67,68]. It has been shown that the exact nature of the hydrophobic support (internal geometry, hydrophobicity, etc,) can greatly alter the lipase functional properties, suggesting that the flexibility of the active center of the lipase can yield different lipase "open" forms [69–73]. The previous studies on effects of immobilized enzyme mineralization were performed utilizing octyl agarose (named agarose C8 in this paper). In this new research effort, we have utilized methacrylate beads bearing octadecyl groups (names Purolite C18 in this paper) to immobilize the enzyme. Together with different groups in the supports (octyl versus decaoctyl), agarose is formed by trunks of agarose [74] while the metacrylated beads are prepared by using porogenic agents during polymerization, that way, the internal geometry of the surfaces will be different: concave or convex. Based on previous literature and the enzyme performance, we can assume that although the enzyme will be immobilized via interfacial activation on both, agarose C8 and methacrylate beads bearing octadecyl, its final conformation may be different. TLL immobilized on a similar support has proved to be a very efficient biocatalyst in the production of biodiesel [75–77], that way, the tuning of the features of this biocatalyst via mineralization may have a great interest. This support has as main difference compared to the previous one a larger particle size. Therefore, in this paper we have analyzed the possibility of tuning (and improving) the hydrolytic activity (versus different substrates) and stability of TLL immobilized on this support, as first step to their utilization in biodiesel production, comparing the effects with those described using agarose C8.

5.2 Materials and Methods

5.2.1 Materials

A TLL solution (20.77 mg protein/mL) was kindly donated by Novozymes Spain (Madrid, Spain). Bradford´s reagent (utilized to calculate the protein concentration [78]), *p*-nitrophenyl-butyrate (*p*-NPB), triacetin, *R*- and *S*-methyl mandelate, triacetin, acetonitrile for HPLC (gradient grade, ≥99.9%), cobalt chloride ($CoCl_2$), copper chloride ($CuCl_2$), and zinc chloride ($ZnCl_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purolite Lifetech® ECR8806F (methacrylate macroporous resin containing octadecyl groups) (Purolite C18) with an average porous diameter of 38.5 nm under dried conditions [79] was kindly donated from Purolite® Ltd. (Wales, UK). Octyl Sepharose® CL-4B (agarose C8) was acquired from GE Healthcare (Uppsala, Sweden), presenting an average porous dimeter of 153 nm in wet conditions [74]. All other reagents were of analytical grade.

5.2.2 Methods

The experiments were repeated 3 times, and the values are given as mean values and standard deviation.

5.2.2.1 Wetting of Purolite C18 beads

1 g of Purolite C18 beads were added to in 5 mL of methanol and mildly stirred to eliminate the air inside the particles [75]. After 1 h, 5 mL of distilled water were added, maintaining the stirring for 15 min. Next, the suspensions were submitted to 10 washes (20 volumes each) with distilled water utilizing vacuum filter, and stowed at 4-6 °C.

5.2.2.2 Immobilization of TLL

Agarose C8 or Hydrated Purolite C18 beads were utilized to immobilize TLL offering 20 mg of protein per g of support. This is over the support loading capacity [79] but it was utilized to guarantee the full lipase coating of the support surface. The immobilization was initialized mixing 100 mL of enzyme solution in 5 mM sodium phosphate at pH 7.0 and 10 g of the support (that means that the enzyme concentration during the immobilization was 2 mg/ml), the immobilization suspension was maintained at room temperature under gentle stirring. The hydrolytic activity in supernatant and reference was periodically determined using *p*-NPB assay activity. After 2 h, the immobilization suspensions were vacuum filtered, washed 10 folds with 20 volumes of distilled water, and kept at 4-6 °C.

5.2.2.3 Modification of immobilized enzyme with metal salt/sodium phosphate

The immobilized enzymes were mineralized mixing $CoCl_2$, $CuCl_2$ or $ZnCl_2$ and sodium phosphate, as described by Guimarães et al. [61]. The biocatalysts (0.5 g) were suspended in 5 mL 10 mM sodium phosphate/125 mM NaCl at pH 7.4, then, a volume of 400 μL of metal salt solution (230 mM) was added and kept at room temperature under mild stirring. After 5 h, the biocatalysts were vacuum filtered, washed 10 folds of distilled water (20 volumes each) and put in storage at 4-6 °C.

5.2.2.4 Thermal inactivation of the different TLL-based biocatalysts

The TLL-based biocatalysts were suspended in 10 mM Tris-HCl (1:10 (w:v) ratio) at pH 7.0 and submitted at 75 °C. To quantify the residual activities, 50 μL of the inactivation suspensions were withdrawn at the desired times. Residual activities were calculated as the hydrolytic activity at the indicated time divided by the initial hydrolytic activity, and this was stated in percentage. Triacetin was utilized to determine the enzyme activity.

5.2.2.5 Enzyme hydrolytic activity assays

One unit of activity (U) corresponds to the conversion of 1 μmol of substrate per minute under the described conditions.

5.2.2.5.1 Hydrolysis of p-NPB

50 μL of a solution/suspension enzyme formulation was mixed to 2.5 mL of 25 mM sodium phosphate at pH 7.0 using a thermostat system at 25 °C under magnetic stirring. 50 μL of 10 mM *p*-NPB dissolved in acetonitrile were added to initialize the reaction. The hydrolyses were performed for 1.5 min. The *p*-nitrophenol released into the medium was quantified using a spectrophotometer. The wave length was 348 nm, as this is the isosbestic point of this compound ($\varepsilon = 5{,}150\ M^{-1}\ cm^{-1}$) [80].

5.2.2.5.2 Hydrolysis of triacetin

3 mL of 50 mM triacetin were prepared in 50 mM of sodium phosphate at pH 7.0. To initialize the reaction, 50 mg of immobilized enzyme were added at 25ºC under gentle stirring. The degree of hydrolysis was quantified by determining the released 1,2 and 1,3 diacetin (the produced 1,2 diacetin undergoes acyl migration giving 1,3 diacetin at pH 7 [81]) in the reaction medium. A Waters 486 chromatograph (Waters, Millford, USA) presenting a Kromasil C18 column (15 cm x 0.46 cm) and a UV/VIS detector (set to 230 nm) were utilized to follow the

degree of conversion (we utilized at least two points, one over 5% and other under 25%). As mobile phase, 85% (v/v) water/15% (v/v) acetonitrile was utilized. The flow rate was 1 mL/min. The retention times were 4 min for 1,2 and 1,3 diacetins (they coeluted under these conditions) and 18 min for triacetin [82].

5.2.2.5.3 Hydrolysis of R- or S-methyl mandelate

3 mL of 50 mM *R*- or *S*-methyl mandelate were prepared in 50 mM sodium phosphate solution at pH 7.0. To start the reaction, 50 mg of TLL-based biocatalysts were added, maintaining the suspension under gentle stirring at 25 ºC. The hydrolysis degree was determined by quantifying the released mandelic acid. A Waters 486 chromatograph (Waters, Millford, USA) with a Kromasil C18 column (15 cm x 0.46 cm) and a UV/VIS detector (set to 230 nm) was employed in the analyses to determine the degree of hydrolysis (two points over 5% and under 25%) [83]. As mobile phase, 65% (v/v) 10 mM ammonium acetate/35% (v/v) acetonitrile at pH 2.8 were utilized. The flow rate was set at 1 mL/min. The retention times were 2.5 min for mandelic acid and 4.2 min for methyl mandelate [84]. Activities ratio was defined as the activity versus the *R*-isomer/activity versus the *S*-isomer.

5.3 Results and Discussion

5.3.1 Preparation of the immobilized and chemically modified TLL

Figure 5.1 shows the immobilization courses of the enzyme immobilized on agarose C8 and Purolite C18. The hydrolytic activity of the enzyme in the presence of inert agarose beads remains unchanged during the whole immobilization process (that is, the enzyme is not immobilized on the inert agarose and remains fully active, as shown in the reference line of the figures). As expected [61,63,67,69], using agarose C8, immobilization was very rapid, with scarce increase in immobilization yield from 0.5 to 2 h (around 55% of the enzyme became immobilized) (around 11 mg/g). Using Purolite C18 immobilization was slower, perhaps due to the higher particle size (300 - 710 μm) that makes diffusion of the enzyme more problematic [85]. After 2 h, around 45% of the enzyme hydrolytic activity was immobilized (9 mg/g of support). Hydrolytic activity versus *p*-NPB of the immobilized enzyme is not included, as in the case of the Purolite C18 the values are almost 0.

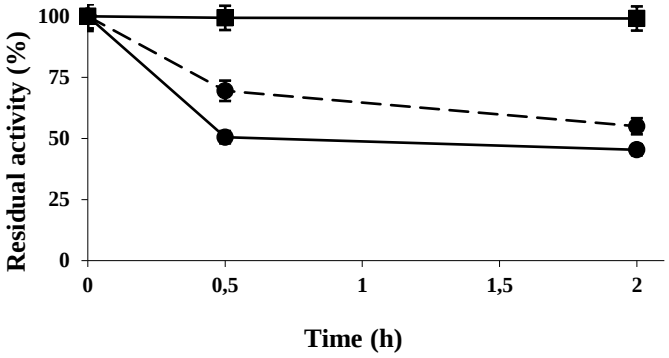

Figure 5.1. Immobilization course of TLL over agarose C8 beads (solid line) and Purolite C18 (pointed line) beads offering 20 mg of enzyme per g of support (10 mL of 2 mg enzyme/mL). Squares: reference (the enzyme under the same conditions of the immobilization suspension but using inert agarose beads) and circles: supernatant. Other specifications are described in Methods.

Table 5.1 shows the hydrolytic activities of both biocatalysts versus triacetin and both isomers of methyl mandelate. The hydrolytic activity versus *p*-NPB was almost 0 using Purolite C18-TLL. Moreover, the amount of immobilized enzyme was slightly lower using Purolite C18 (9 mg/g) compared to agarose C8 (11 mg/g) (see Figure 5.1). However, the hydrolytic activity of Purolite C18-TLL was more than 4-fold higher than that of the agarose C8 preparation using the triacetin assay. In the case of methyl mandelate, the effects of the support depended on the used enantiomer. While the hydrolytic activity versus the *R*-isomer increased using Purolite C18-TLL (by almost 1.7-fold), the hydrolytic activity is more than 2 times higher using agarose C8-TLL. As a result, the hydrolytic activities ratio with these isomers increased from 1.9 using agarose C8-TLL to 7.3 using Purolite C18-TLL. Considering that the diffusional and steric restrictions must be identical for both isomers, this suggests that the differences in hydrolytic activities are really related to different enzyme conformations [4]. That way, the immobilization on different supports produced biocatalysts with very different hydrolytic activities and specificities.

Table 5.1. Activity of the prepared biocatalysts in the hydrolysis of different substrates under the defined conditions. Experiments were conducted as described in Methods.

Biocatalysts	Activity (U/g)		
	Substrate		
	Triacetin	*R*-Mandelate	*S*-Mandelate
Agarose C8-TLL	115.7 ± 5.9	1.3 ± 0.1	0.70 ± 0.05
Purolite C18-TLL	548.7 ± 25.9	2.2 ± 0.1	0.30 ± 0.02

Figure 5.2 shows that TLL immobilized on Purolite C18 was significantly more stable than the enzyme immobilized on agarose C8 (following the hydrolytic activity with triacetin), that is already considered as a very stable biocatalyst [67,68]. That way, Purolite C18-TLL seemed to offer some advantages on stability and hydrolytic activity compared to agarose C8-TLL, and in part this may be related to a different enzyme conformation. Considering that the immobilization conditions define the properties of this enzyme on this kind of supports [86], this result could be expected.

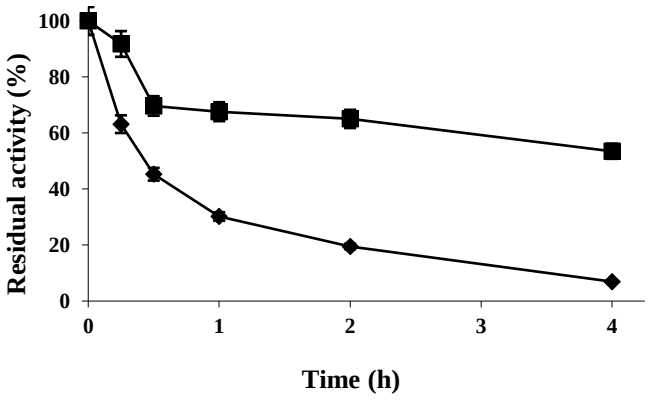

Figure 5.2. Thermal inactivation courses of agarose-C8l-TLL and Purolite C18-TLL. Other specifications are described in Methods. Agarose C8-TLL (rhombus) and Purolite C18-TLL (squares).

Next, we have intended to further improve these enzyme features via mineralization of Purolite C18-TLL [61–64].

5.3.2 Mineralization of Purolite C18-TLL

The biocatalyst was incubated with sodium phosphate and different metal chloride salts, to get metal phosphate mineralization of the immobilized enzymes [61–64]. Figure 5.3 shows the color that the biocatalyst acquired after mineralization. The supports are not transparent like agarose, and they have an initial slightly grey color, that way the zinc phosphate mineralization does not produce an obvious change in the color. Copper (blue) and cobalt (slight pink) phosphates mineralization produced an obvious change in the color of the biocatalyst.

Figure 5.3. Purolite C18 TLL (1) unmodified modified with (2) $ZnCl_2$/sodium phosphate, (3) $CuCl_2$/sodium phosphate and (4) $CoCl_2$/sodium phosphate. Other specifications are described in Methods.

5.3.2.1 Effect of the mineralization on enzyme hydrolytic activities

Using agarose C8-TLL [61] and diverse blocked agarose C8-vinyl sulfone-TLL [63], the mineralization greatly affected the enzyme properties, thus as using the commercial TLL biocatalyst [64]. Table 5.2 shows the hydrolytic activity of the differently modified biocatalysts. When the Purolite C18 biocatalyst was mineralized with zinc phosphate, its hydrolytic activity versus triacetin increased by 23.2%. Due to the high hydrolytic activity of the biocatalysts, it is not discarded that the effects may be even higher but masked due to substrate diffusion limitations [87–89] and pH gradients [4,90–93]. The same treatment increased the hydrolytic activity of the agarose C8-TLL by around 38.9%, but its activity was still less than a quarter of the activity of the Purolite C18-TLL. The modification with copper salt increased the hydrolytic activity of Purolite C18-TLL similarly to the zinc salt, while using agarose C8-TLL, this treatment produced no change in the hydrolytic activity with this substrate. Cobalt salt mineralization produced the most active biocatalyst, almost 720 U/g using Purolite C18-TLL (an increase in the hydrolytic activity by 30.5%), while a minor increase in hydrolytic activity

was found using agarose C8-TLL (3.2%). These changes in hydrolytic activity suggest that the different mineralizations, acting on different enzyme conformations, can produce very different effects. For this substrate, the changes are more positive using Purolite C18-TLL than agarose C8-TLL, even being both biocatalysts based in the same immobilization procedure: lipase interfacial activation [68].

For the methyl mandelate isomers, the situation is again different for Purolite C18-TLL and agarose C8-TLL. Focusing on the *R*-isomer, the zinc salt treatment increased the hydrolytic activity of Purolite C18-TLL by 14.0% (while using agarose C8-TLL this activity decreased to 69.2%), the copper salt treatment produced a similar increase in hydrolytic activity of Purolite C18-TLL while this activity was maintained using agarose C8-TLL, the cobalt phosphate treatment produced an increase of the hydrolytic activity of 9.1% using Purolite C18-TLL while it decreased to 69.2% using the other biocatalyst. Using the *S*-isomer, all mineralizations decreased the hydrolytic activity of both biocatalysts, but the decrease is much more significant using Purolite C18-TLL (to 33.3% using copper salt) than the agarose C8-TLL (to 71.4% using zinc or copper salts). That way, while the initial Purolite C18-TLL biocatalyst presented ratio of activities in hydrolysis of R- to S-enantiomers of 7.3, all mineralizations increased this ratio: using zinc salt, this ratio was 12.5, using copper salt, it was 25 and using cobalt salt, this ratio was 12. For agarose C8-TLL the changes in this ratio are smaller and some are negative, moving from the initial 1.85 to 1.8, 2,6 or 1.5, respectively.

That way, the effects of the mineralization of immobilized TLL hydrolytic activity and specificity strongly depended on the support. These suggested that the enzyme not only had different initial conformation in both biocatalysts, but that the changes induced by the mineralization were also different [59–64].

Table 5.2. Relative activity (calculated as the ratio activity of the modified biocatalyst/activity of the unmodified biocatalysts in percentage) of different TLL based biocatalysts in the reaction of substrate hydrolysis under the defined conditions. Experiments were conducted as described in Methods.

Biocatalysts	**Relative activity (%)**								
	$Zn_3(PO_4)_2$ modification			**$Cu_3(PO_4)_2$ modification**			**$Co_3(PO_4)_2$ modification**		
	Triacetin	***R*-Mandelate**	***S*-Mandelate**	**Triacetin**	***R*-Mandelate**	***S*-Mandelate**	**Triacetin**	***R*-Mandelate**	***S*-Mandelate**
Agarose C8-TLL	138.9 ± 6.7	69.2 ± 3.9	71.4 ± 2.8	100.0 ± 5.1	100.0 ± 3.4	71.4 ± 4.3	103.2 ± 4.2	69.2 ± 3.5	100.0 ± 5.1
Purolite C18-TLL	123.2 ± 3.7	114.0 ± 5.1	66.7 ± 2.5	120.2 ± 3.6	114.0 ± 4.4	33.3 ± 1.6	130.5 ± 3.9	109.1 ± 6.1	66.7 ± 2.9

5.3.2.2 Effect of the mineralization on enzyme stability

Next, we have studied the effect of the different treatments on the stability of the Purolite C18-TLL. In the previous study using agarose C8 beads [61], zinc salt treatment was found to be stabilizing, while cobalt and copper salts treatments slightly destabilized the enzyme. Figure 5.4 shows that while copper and cobalt salts treatments produced a destabilization of the enzyme (more relevant than using agarose C8-TLL), the zinc salt treatment produced some stabilization. This stabilization was smaller than the one described using agarose C8-TLL [61]. However, as showed in Figure 5.5, the stability of agarose C8-TLL-zinc phosphate was slightly lower than that of Purolite C18-TLL-zinc phosphate. That way, the enzyme mineralization effect on the enzyme stability depends on the metal used, and also on the utilized support. The higher stabilization caused by the mineralization of agarose C8-TLL greatly decreased the high difference in the initial stability when compared to Purolite C18-TLL.

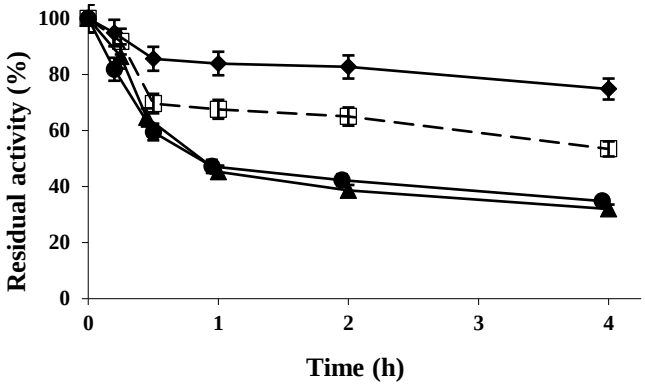

Figure 5.4. Thermal inactivation courses of different Purolite C18-TLL biocatalysts. Other specifications are described in Methods. Unmodified biocatalyst (open squares and dotted line); biocatalyst modified with $ZnCl_2$/sodium phosphate (solid rhombus and solid line); $CoCl_2$/sodium phosphate (solid triangles and solid line); $CuCl_2$/sodium phosphate (solid circles and solid line).

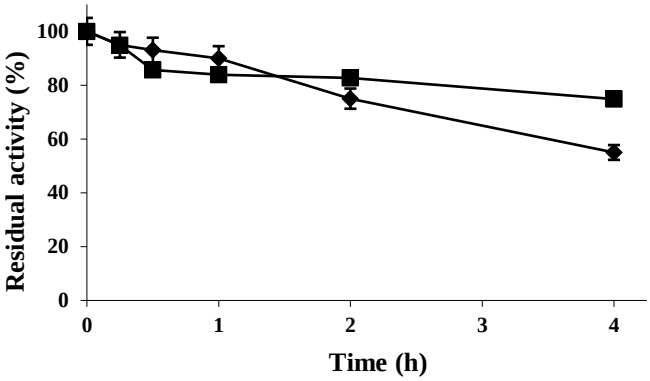

Figure 5.5. Thermal inactivation courses of agarose C8-TLL-ZnP (rhombus) and Purolite C18-TLL-ZnP (squares). Other specifications are described in Methods.

5.4 Conclusions

The immobilization of TLL on Purolite C18 produced a biocatalyst with properties quite different to those obtained utilizing agarose C8, suggesting that the enzyme structure may be quite different. Purolite C18-TLL was almost inactive in hydrolysis of *p*-NPB, while it was much more active in the hydrolysis of other substrates (triacetin or methyl mandelate) than the agarose C8 biocatalyst. Moreover, Purolite C18-TLL presented a higher hydrolytic activity with *R*–methyl mandelate.

The mineralization of Purolite C18-TLL also produced diverse effects compared to the mineralization of agarose C8-TLL. In terms of hydrolytic activity, it is generally more positive now than with the agarose biocatalyst. However, Purolite C18-TLL lost more stability using cobalt or copper phosphates treatment than using agarose C8-TLL, while its stability increased to a smaller degree using zinc salt treatment.

That way, the results suggest that even having an enzyme immobilized using the same protocol (interfacial activation), the differences in the support can generate different enzyme conformations and thus, the enzyme mineralization can present diverse effects on enzyme features. Only after analyzing the mineralization effects on the exact process, we can assure that the changes may be positive or negative. Only an empiric analysis can show the effects.

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CHAPTER 6
THE IMMOBILIZATION PROTOCOL GREATLY ALTERS THE EFFECTS OF METAL PHOSPHATE MODIFICATION ON THE ACTIVITY/STABILITY OF IMMOBILIZED LIPASES

Mineralization of immobilized enzymes has showed to couple the advantages of both processes. Here, the influence of the immobilization protocol on the effects of mineralization has been investigated. The lipases from *Thermomyces lanuginosus* and *Candida rugosa* were immobilized on octyl-, vinyl sulfone (VS) octyl (blocked with different nucleophiles) and glutaraldehyde- (at different pH values) agarose beads. The stability, activity and specificity of the biocatalysts were very different, both the differently blocked VS-biocatalysts and the glutaraldehyde biocatalysts prepared at different pH. All biocatalysts were submitted to mineralization using different metals. The activity, specificity and stability effects of the mineralization strongly depended on the enzyme and on the immobilization protocol. For the same enzyme, a mineralization protocol could be negative, positive or present no effect depending on the enzyme immobilization procedure and substrate. In the best cases, activity could be increased by a two-fold factor, while stability was significantly improved in many instances. These results highlight the great potential of mineralization of immobilized enzymes to improve their properties, as well as the great interactions that immobilization protocol and mineralization can exhibit. The combination of both methodologies greatly increases the possibilities to find a biocatalyst that can be suitable for a specific process.

6.1 Introduction

Lipases have been utilized in many different reactions because they present a broad specificity, are stable and active in a wide range of conditions and reaction media and can catalyze many different reactions [1–9] (including promiscuous ones) [10–12]. That way, they can be useful in very different applications [13–19]. Unfortunately, in many instances, the properties of lipases (as other enzymes) do not fit the requirements of an industrial catalyst, and their catalytic performance in industrial reactions (in many instances using substrates and conditions far from the physiological ones) is inadequate [20]. Nowadays, there are many

strategies to solve some of the problems of lipases, their properties may be improved using metagenomics (to use the most adequate available enzyme) [21–24], site-directed mutagenesis [25–29] or directed evolution [30–33]. Another problem of enzymes is their water solubility, which can complicate their recovery. This makes their reuse and reaction control complex. Enzyme immobilization was the answer to solve this enzymes drawback, and nowadays it has been revealed as a tool to greatly improve many enzyme features, such as stability, selectivity, specificity, inhibition, etc. [34–45]. It can be also coupled with enzyme purification [46]. However, these benefits may be reached only if the immobilization protocol is carefully designed [47].

The lipase catalytic mechanism (interfacial activation) [3,48–50] causes their active center to be very flexible, and that way, lipase features are especially easy to be tuned by any of the previously described strategies, increasing the prospects of getting biocatalysts adequate for a specific process [51–56].

Among the lipase immobilization processes, the preparation of enzyme/metal salts nanoflowers has been established as a strategy that can give heterogeneous lipase biocatalysts and, at the same time, improve enzyme activity and stability in certain cases [57–61]. This strategy is based on the function of some residues of the enzyme surface as crystal nucleation points, generating crystal structures that have the appearance of the petals of a flower, or even of the whole flower (hence the name) after full growth. Although bearing an undeniable interest, the poor mechanical resistance of these nanoflowers is delaying their implementation. To solve this problem, the trapping of the enzyme nanoflower in structures bearing better mechanical resistance has been proposed, together with the use of magnetic materials to facilitate their handling [62–69].

Our research group recently proposed performing the modification by metal phosphate on already immobilized enzymes as an alternative to solve the nanoflowers fragility while maintaining some of the mineralization benefits [70]. If the enzyme mineralization was performed on previously immobilized enzymes, the researcher can select the support based on its mechanical resistance (and reap the benefits achieved during enzyme immobilization), avoiding the difficulties derived from the management of the small and fragile nanoflowers. Moreover, the mineralization of immobilized enzyme couples the positive effects of enzyme mineralization during nanoflowers production with the benefits of enzyme immobilization in preexisting solids

[70]. In this first report, several enzymes were immobilized on octyl agarose beads via interfacial activation [71] and modified with diverse metal phosphates. It was found that the effects of the metal phosphate modification were clearer and more positive using highly loaded biocatalyst, suggesting that enzyme crowding could facilitate some of the positive effects of enzyme mineralization [70]. The effects depended on the nature of both enzyme and metal, in some instances enabling very significant stabilizations, and optimal results were achieved using the metal phosphate in the modification of already immobilized enzymes. The incubation with only sodium phosphate or only metal chloride, as well as the immobilization on previously modified supports (the supports could be modified by using metal phosphates) which produced significantly reduced effects [70]. This modification of the support complicates finding any structural proof of the reasons for the great modification of the enzyme properties upon mineralization. However, the important alteration of the functional properties of the enzymes after their mineralization presented a significant interest. The study was later extended to the use of diverse commercial biocatalysts [72]. The immobilized enzyme mineralization cannot produce a tridimensional nanoflower, as the enzymes will be located on a flat planar surface, but the previous results suggest that the positive effects of the building of nanoflowers may be, at least partially, achieved using this solid-phase strategy. However, we cannot talk of nanoflowers, as these tridimensional structures will never be achieved.

There are some reports showing that the immobilization protocol can greatly alter the effect of some chemical or physical modifications of immobilized enzymes [73–77]. The mineralization of the commercial lipase biocatalysts offered very different results to those described using the octyl-lipases, suggesting that this could be the case for enzyme mineralization [72].

In a very recent paper [78], the authors have shown that the nucleation induced by a protein during the production of metal–organic frameworks produced using 2-methylimidazole and zinc acetate strongly depended on the tridimensional structure of the protein, origin salt crystals with very different structures.

In this new communication, we have studied the possible influence of the immobilization protocol on the effects that the metallization of the immobilized lipases can present. For this reason, we will compare the results obtained when modifying with metal phosphate enzymes

immobilized using octyl agarose [71,79] with other two immobilization techniques whose versatility permits to tailor the immobilized enzyme features.

The first one is the immobilization of the lipases on amino-glutaraldehyde agarose beads [80]. The main immobilization cause on these supports is the ionic exchange of the enzyme on the support surface, followed by the establishment of some covalent bonds between the primary amino bonds of the adsorbed enzyme and the glutaraldehyde groups in the support [81–83]. As it has been reported that the pH in the immobilization process greatly affects the enzyme features after its immobilization, we performed the immobilization at pH 5 and 8 [84–89] (Figure 6.1).

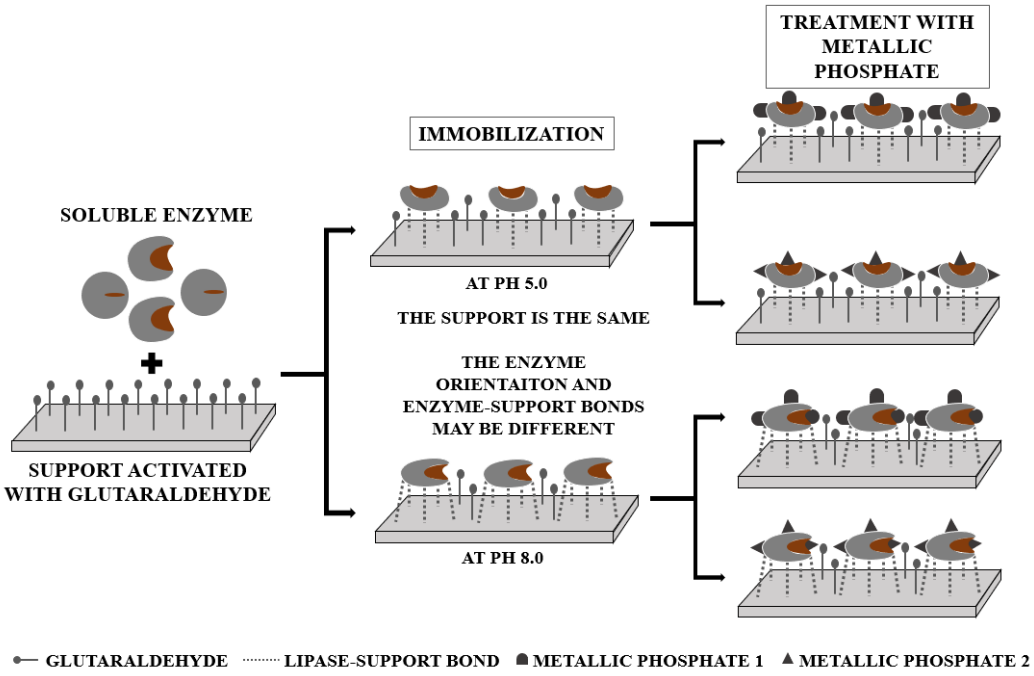

Figure 6.1. Schematic representation of enzyme immobilization on glutaraldehyde-activated support at different pH values.

The second one is the immobilization of the lipases on agarose beads simultaneously presenting octyl and vinyl sulfone groups, an immobilization protocol that produces some of the most stable formulations described in the literature for the employed enzymes [90]. In this case, the enzyme is first immobilized via interfacial activation, and after changing the conditions, some groups of the enzyme (e.g., lysine, histidine, cysteine (Cys), and tryptophan) [91] can react with the vinyl sulfone groups [90]. This strategy has a final step that requires the blocking of the remaining reactive groups of the support with a nucleophile. This step enables tailoring of the

enzyme-support interactions by using reagents with very different physical properties [90,92–95] (Figure 6.2). Moreover, it greatly alters the functionality of the enzymes (enzyme stability and activity) and this has been correlated to the promotion of different enzyme structures [96]. Furthermore, it has been shown how the different enzyme-support interactions promote different inactivation pathways [96], similarly to the enzyme rigidification of different enzyme areas [97]. That way, playing with the blocking reagent, we will have an immobilized enzyme collection with exactly the same enzyme distribution in the support particle, orientation with respect to the support surface (they are interfacially activated) and number of enzyme support bonds, but bearing a different structure and functional properties.

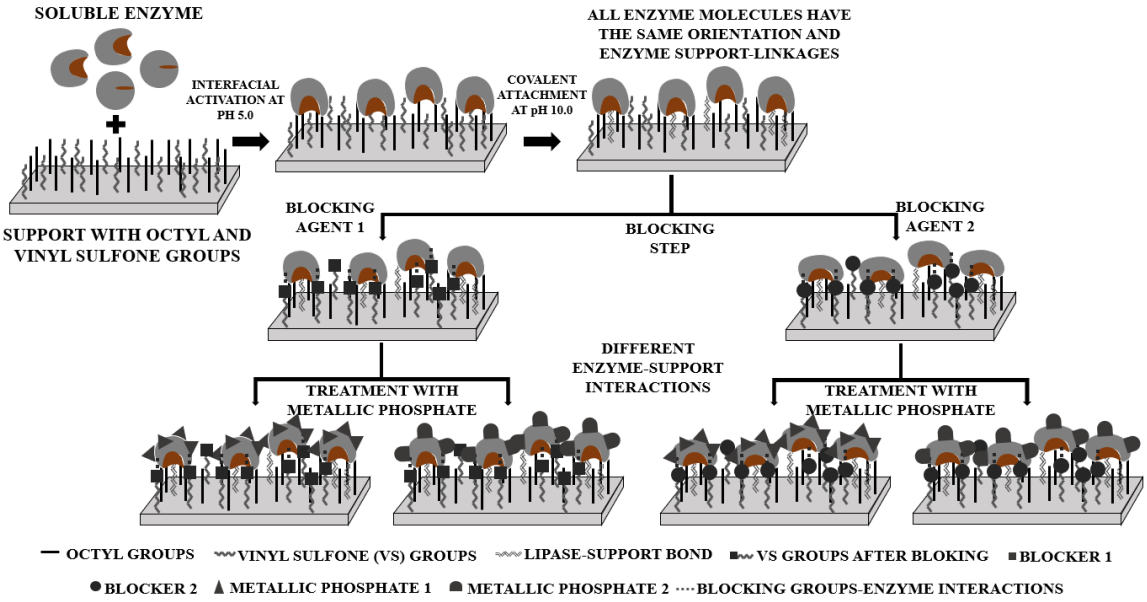

Figure 6.2. Schematic representation of enzyme immobilization on octyl and divinyl sulfone groups heterofunctionalized support.

Both immobilization strategies will permit to analyze if the enzyme structure or orientation regarding the support (or both) may alter the effects of the mineralization as it has been found for other modifications. As model enzymes to analyze these possible immobilization protocol/mineralization interactions, we have selected the two enzymes that were more significantly stabilized by mineralization when using octyl agarose [70], both very popular ones: the lipases from *Thermomyces lanuginosus* [98] and from *Candida rugosa* [99–102]. The use of at least two different enzymes permitted to get more generalized conclusions.

6.2 Materials and Methods

6.2.1 Materials

A TLL formulation with 20.77 mg protein/mL (kindly donated by Novozymes Spain (Madrid, Spain)) was utilized in this paper. Lipase from *Candida rugosa* (CRL) was acquired from Sigma-Aldrich (St. Louis, MO, USA), it was a solid powder with 3.12 mg protein/g of powder. Bradford´s reagent (utilized to calculate the protein concentration [103]), *p*-nitrophenyl-butyrate (*p*-NPB), triacetin, *R*- and *S*-methyl mandelate, acetonitrile for HPLC (gradient grade, ≥99.9%), glycine (Gly), aspartic acid, ethylenediamine (EDA), cysteine, Tris(2-aminoethyl)amine (TrisAEA), glutaraldehyde (GA) solution (25% in H_2O), $CoCl_2$, $CuCl_2$ and $ZnCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Divinyl-sulfone (DVS) was purchased from Thermo Fisher Scientific Spain (Madrid, Spain). Octyl Sepharose® CL-6B was acquired from GE Healthcare (Uppsala, Sweden). All other reagents were of analytical grade.

6.2.2 Methods

All experiments were performed at least by triplicate, and the values are presented as mean values and standard deviation. Protein concentration was determined using Bradford method [103].

6.2.2.1 Preparation of octyl-vinyl sulfone agarose beads

The octyl-vinyl sulfone agarose support was prepared following the methodology described by Albuquerque et al [90]. 1 g of octyl agarose was added to 20 mL of 350 mM divinyl-sulfone prepared in 333 mM sodium carbonate at pH 11.5, having a vinyl sulfone group in the support. Activation of the support was carried out at room temperature under gentle stirring for 2 h. Afterwards the support was vacuum filtered, washed 5 folds with 20 volumes of distilled water and stored at 4-6 °C.

6.2.2.2 Preparation of the glutaraldehyde-agarose support

A mass of 1 g of amine agarose (prepared according to the methodology described by Fernández-Lafuente et al. [104]) was added to 10 mL of 10% glutaraldehyde prepared in 200 mM sodium phosphate at pH 7. The activation process took place at room temperature under gentle

stirring for 16 h. Then, the support was vacuum filtered, washed 5 times with 20 volumes of distilled water and stored at 4-6 °C.

6.2.2.3 Immobilization of lipases

In all cases, activity immobilization yield (percentage of offered enzyme activity that is immobilized on the support) and expressed activity (percentage of observed activity with respect to the expected one from the immobilization yield) are utilized to define the immobilizations [105]. The mineralization only affects the expressed activities. The enzyme load is calculated from the offered activity and the enzyme activity that remains in the supernatant. Activity is not released from the support during the washings, but around 100 mg of support is lost in the filters during the washing steps (this is a problem in the lab that may not occur using other washing devices). In fact, the covalent immobilizations make that not protein bands may be detected in SDS-PAGE experiment (not shown results) [90].

6.2.2.3.1 Immobilization of lipases on octyl-agarose beads

A mass of 10 g of support was added to 100 mL of enzyme solution prepared in 5 mM sodium phosphate at pH 7.0 (enzyme loads: TLL: 20 mg/g and CRL: 30 mg/g). The immobilization was conducted at room temperature under gentle stirring for 2 h (Agimatic-S (JP Selecta) (Barcelona, Spain)). The enzyme activity in the supernatant, suspension and a reference (where octyl agarose was substituted by inert agarose) were quantified using *p*-NPB assay throughout the immobilization course. Then, the suspensions were filtered and the biocatalysts were washed 5 folds with 20 volumes of distilled water, and stored at 4-6 °C.

6.2.2.3.2 Immobilization of lipases on octyl-vinyl sulfone agarose beads

A mass of 10 g of support was added to 100 mL of enzyme solution prepared in 5 mM sodium acetate at pH 5.0 (enzyme loads: TLL: 20 mg/g and CRL: 30 mg/g) [90]. In this step, pH 5.0 was used to favor interfacial activation as the main cause for enzyme immobilization [90]. The immobilization was conducted at room temperature under gentle stirring for 2 h (Agimatic-S (JP Selecta) (Barcelona, Spain)). The enzyme activity in the supernatant, suspension and a reference were quantified using *p*-NPB assay throughout the immobilization course. Then, the biocatalysts were vacuum filtered, washed 5 times with 20 volumes of distilled water, and

recovered. The biocatalysts were resuspended in 100 mM sodium carbonate at pH 10.0 in a biocatalyst/buffer ratio of 1/10 (w/v). This step was used to favor the formation of the enzyme-support covalent bond(s). The reaction was conducted at room temperature for 24 h. Afterwards, the biocatalysts were vacuum filtered and recovered. Finally, 1 g of biocatalyst was added to 10 mL of 2 M blocking agent (glycine, aspartic acid, cysteine, EDA, TrisAEA) at pH 10.0. This step was carried out at room temperature under gentle stirring for 48 h in order to modify the residual vinyl sulfone moieties. Then, the biocatalysts were vacuum filtered, washed 10 folds with 20 volumes of distilled water, and stored at 4-6 °C.

6.2.2.3.3 Immobilization of lipases on amino-glutaraldehyde (Glu)-agarose beads

A mass of 10 g of support was added to 100 mL of enzyme solution prepared in 5 mM sodium phosphate at pH 7.0 (enzyme loads: TLL: 20 mg/g and CRL: 30 mg/g). The immobilization was conducted at room temperature under gentle stirring for 2 h (Agimatic-S (JP Selecta) (Barcelona, Spain)). The immobilization was followed measuring *p*-NPB activity of the supernatant, suspension and reference solutions. After immobilization, the biocatalysts were filtered, washed 5 folds with 20 volumes of distilled water, vacuum dried and stored at 4-6 °C by a minimum of 48 h before any use.

6.2.2.4 Modification of immobilized enzyme with metallic salt/phosphate

The immobilized enzymes were modified with metallic salt/phosphate following the methodology described by Guimarães et al. for octyl agarose immobilized lipases [70]. 1 g of immobilized enzyme was suspended in 10 mL of 10 mM sodium phosphate/ 125 mM NaCl at pH 7.4 and, then, 400 µL of 230 mM of the corresponding metallic salt solution was added. The enzyme treatment was conducted at room temperature under gentle stirring for 5 h (Agimatic-S (JP Selecta) (Barcelona, Spain)). After modification, the suspension was filtered and the biocatalysts were washed 5 folds with 20 volumes of distilled water, and stored at 4 °C.

6.2.2.5 Thermal inactivations of the different lipase preparations

In a standard experiment, 1 g of immobilized biocatalyst was suspended in 10 mL of 10 mM Tris-HCl at pH 7.0 and incubated at different temperatures (immobilized TLL: 75 °C and immobilized CRL: 70 °C). Periodically, samples of 50 µL of the inactivation suspensions were

collected to determine their residual activities (current activity divided by the initial one in percentage). The experiments were performed employing *p*-NPB as substrate.

6.2.2.6 Enzyme activity assays

One unit of activity (U) was defined as the amount of enzyme that hydrolyzes one µmol of substrate per minute under the described conditions.

6.2.2.6.1 Hydrolysis of p-NPB

A volume of 50 µL of 50 mM *p*-NPB dissolved in acetonitrile was added into a mixture of 2.5 mL of 25 mM sodium phosphate at pH 7.0. The experiments were performed in a Jasco V-730 spectrum (Madrid, Spain) with a stirring system and temperature regulation. To initialize the reaction, a volume of 50 µL of enzyme solution or immobilized enzyme suspension was added. The reaction was carried out at room temperature under magnetic stirring. The quantification of hydrolytic activity was determined by the increase in absorbance at 348 nm produced by the release of p-nitrophenol (*p*-NP) (isosbestic point of *p*-NP, its ε under these conditions is 5,150 M^{-1} cm^{-1}) for 300 seconds [106].

6.2.2.6.2 Hydrolysis of triacetin

125 mg of immobilized enzyme were added to 10 mL of 50 mM of triacetin prepared in 50 mM of sodium phosphate at pH 7.0. Hydrolysis was carried out at room temperature under gentle stirring (Agimatic-S (JP Selecta) (Barcelona, Spain)), and quantified by detection of 1,2 and 1,3 diacetin (under these conditions, the produced 1,2 diacetin suffers acyl migration giving 1,3 diacetin) [107]. The degree of conversion (two points over 5% and under 25%, to ensure linearity and minimize experimental error caused by the initial acid content of the samples) was calculated by HPLC in a Waters 486 chromatograph (Waters, Millford, USA) equipped with a UV/VIS detector (set to 230 nm) using a Kromasil C18 column (15 cm x 0.46 cm) with a mobile phase composed of 85% (v/v) of water and 15% (v/v) of acetonitrile with a flow rate of 1 mL/min. The retention times were 4 min for 1,2 and 1,3 diacetins (under these conditions. they eluted at the same retention time) and 18 min for triacetin [108].

6.2.2.6.3 Hydrolysis of R- or S-methyl mandelate

250 mg of immobilized lipase were added to 10 mL of 50 mM *R*- or *S*-methyl mandelate in 50 mM sodium phosphate solution at pH 7.0. Hydrolysis was carried out at room temperature under gentle stirring (Agimatic-S (JP Selecta) (Barcelona, Spain)), and the substrate and product concentrations were determined by HPLC using a Waters 486 chromatograph (Waters, Millford, USA). The equipment features a UV/VIS detector (set to 230 nm) and a Kromasil C18 column (15 cm x 0.46 cm). The mobile phase was 10 mM ammonium acetate and acetonitrile (65%-35% (v/v)) at pH 2.8 with a flow rate of 1 mL/min. The retention times were 2.5 min for mandelic acid and 4.2 min for the *R*- or *S*-methyl mandelate. Conversions under 25% (two points over 5% and under 25%, to ensure linearity and minimize experimental error caused by the initial acid content of the samples) were used to calculate enzyme activity [94]. Activities ratio was defined as the activity versus the *R*-isomer/activity versus the *S*-isomer. This reaction was utilized to check the operational stability of the biocatalysts. For this purpose, a syringe with a silica filter at the bottom to prevent the leakage of the biocatalyst was used, utilizing 1 g of biocatalysts and 10 mL of 10 mM ester solution in 200 mM sodium phosphate at pH 7 for 2 h. After each cycle, the biocatalysts were washed 3 times 10 mL with distilled water before reloading the substrate solution. Activity was determined when less than 20% of the ester had been hydrolyzed.

6.3 Results and Discussion

6.3.1 The case of TLL biocatalysts

6.3.1.1 Immobilization of TLL in the different supports

First, TLL and CRL have been immobilized on octyl-, octyl-VS and amino-glutaraldehyde-agarose beads. Our objective was to fully coat the support surface with enzyme molecules, as this was found to favor the effects of the mineralization on the enzyme properties [70]. That way, in all cases, we utilized an excess of enzyme on the immobilization. Figure A6.1 shows the immobilization course of TLL on octyl agarose. Around 10 mg of enzyme extract/g of support are immobilized (50% of the offered activity). Using octyl-VS agarose beads (Figure A6.2), the immobilization rate and yield are very similar. The immobilization of the enzyme on

glutaraldehyde was performed at pH 5 and 8 (Figure A6.3). Immobilization was almost total in both cases.

Table 6.1 summarizes the activity of the different TLL preparations versus *p*-NPB, triacetin and *R*- and *S*-methyl mandelate. Starting with TLL-octyl, maximum activity is observed using *p*-NPB, being the activity versus triacetin more than 10 folds lower. The activity versus mandelate esters is around 100-fold lower than versus triacetin, preferring the hydrolysis of the *R*-isomer by almost a 2-fold factor.

Table 6.1. Mass activity (immobilized biocatalyst) of different TLL biocatalysts with different substrates: 50 mM *R*- or *S*-methyl mandelate (pH 7 and 25 °C), 50 mM of triacetin (pH 7 and 25 °C) and 1 mM of *p*-NPB (pH 7 and 25 °C). Experiments were performed as described in Methods.

Biocatalyst	**Activity (U/g)**			
	***p*-NPB**	**Triacetin**	***R*-Mandelate**	***S*-Mandelate**
TLL-octyl	1390.9 ± 25.0	110.9 ± 5.4	1.3 ± 0.06	0.7 ± 0.03
Gly-TLL-VS-octyl	3279.0 ± 20.6	102.1 ± 4.1	1.2 ± 0.05	0.4 ± 0.02
EDA-TLL-VS-octyl	3038.8 ± 51.7	83.6 ± 3.7	1.4 ± 0.02	0.3 ± 0.01
TrisAEA-TLL-VS-octyl	3000.6 ± 28.3	94.0 ± 4.9	0.6 ± 0.02	0.07 ± 0.003
Asp-TLL-VS-octyl	3109.6 ± 132.4	94.0 ± 3.7	0.5 ± 0.02	0.4 ± 0.02
Cys-TLL-VS-octyl	2755.4 ± 98.1	64.1 ± 2.2	0.5 ± 0.02	0.2 ± 0.01
TLL-Glu (pH 5.0)	37.6 ± 2.2	2.7 ± 0.1	10.6 ± 0.5	10.6 ± 0.5
TLL-Glu (pH 8.0)	15.6 ± 0.1	2.0 ± 0.1	9.1 ± 0.4	13.8 ± 0.7

Analyzing the VS-octyl biocatalysts, clear differences may be found among the different biocatalysts as a function of the blocking agent utilized as reaction end point. The Gly blocked biocatalysts presented over 2.3 more activity versus *p*-NPB than the octyl preparation, while their activity decreased by less than 10% the activity of this biocatalyst versus triacetin. In the

mandelate esters, the activity was almost maintained using the *R*-isomer, while it decreased using the *S*-isomer. That way the activities ratio versus both isomers became 3. If the blocking is performed using EDA, activity versus *p*-NPB is slightly lower than in the other case, while the decrease in the activity versus triacetin was more significant (to around 75% of that of the octyl preparation). Regarding the methyl mandelate isomers, the activity versus the *R*-isomer slightly increased while the activity versus the *S*-isomer decreased compared to the octyl preparation, the activities ratio became almost 5. Using TrisEAE as blocking agent, the activity versus *p*-NPB was similar to the case of EDA, higher using triacetin, and the clearest difference is using the mandelic esters. The activity versus the *R*-isomer decreased while the activity versus the *S*-isomer was maintained (compared to the TLL octyl biocatalyst), the resulting activities ratio is lower than 1. Using Asp to block the biocatalyst, activity versus *p*-NPB was 2.2-fold higher than using the octyl preparation, the activity was 85% using triacetin and decreased more versus the *R*- than versus the *S*-methyl mandelate, the activities ratio become 1.25. Blocking using Cys gave an activity doubling that of the TLL-octyl preparation versus *p*-NPB, maintaining 60% of the activity versus triacetin, and decreased the activity more versus *S*-methyl mandelate than versus the *R*-isomer, giving an activities ratio of 2.5. That way, the functional properties of all the biocatalysts were quite different depending on the blocking agent, as it has been previously reported [90,92–94] altering in a very significant way the enzyme specificity and enantiospecificity. Considering that all biocatalysts have the same enzyme distribution on the support, the same orientation (the enzyme is interfacially activated) and the same number of enzyme-supports bonds, the differences should be related to the only difference among the biocatalysts: the different support surface moieties. That way, the different interactions between enzyme and support surface should be the reason for these different enzyme features. Comparing the vinyl sulfone- octyl biocatalysts to the octyl biocatalyst, differences are extended to possible distortions caused on the enzyme surface by the covalent bonds. Some role of the immobilization pH cannot be discarded, as it has been reported to alter also the functional properties of lipases immobilized on hydrophobic supports, being also associated to the creation of different enzyme structures [96]. In any case, the objective of having a collection of biocatalysts with very different functional properties has been accomplished.

Using the glutaraldehyde preparations, again great differences could be found depending on the immobilization pH value. The activity versus *p*-NPB of the enzyme immobilized at pH 8 is

2.5-fold lower than that of the enzyme immobilized at pH 5. Although the support enzyme loading is double than that when using the octyl preparation (because we use agarose 6BCL and immobilization yields is next to 100% (Figure A6.3)), the activity versus *p*-NPB is much lower than those described for the hydrophobic supports (near 100-fold in the most extreme case). Using triacetin, activity is again much lower than in the other biocatalysts (by almost a factor of 50), but the activities of both glutaraldehyde biocatalysts become more similar (1.35-fold higher for the enzyme immobilized at pH 5). However, the activity versus the methyl mandelate isomers is much higher than that found for the octyl biocatalysts (by almost 7-fold). In fact, the glutaraldehyde TLL biocatalysts presented more activity versus methyl mandelate than versus triacetin, in opposition with the other preparations. The enzyme immobilized at pH 5 did not discriminate between both isomers, while the enzyme immobilized at pH 8 preferred the *S*-isomer (with an activities ratio of around 0.65), as opposed to most of the octyl preparations. That way, all the TLL biocatalysts have fully different specificities, in agreement with previous reports [74,82].

Another functional feature that is modulated by the immobilization protocol and blocking step is the stability of the immobilized enzyme [84,86,87,89,90,92–94]. Figure 6.3 shows the inactivation courses of all the prepared biocatalysts. TLL-octyl presented a stability similar to that of the enzyme immobilized at pH 5 on glutaraldehyde, while the enzyme immobilized at pH 8 on glutaraldehyde was more stable. It should be considered that TLL has been immobilized in absence of detergent, that way, very likely, both glutaraldehyde biocatalysts may have mainly lipase dimers, with the open form of one molecule of TLL stabilized by another open lipase [109–112]. Furthermore, at pH 8, the possibilities of having a more intense multipoint covalent attachment increased, explaining the higher stability of this biocatalyst [42]. As previously reported [90,96], all the octyl-VS biocatalyst improved the stability compared to the octyl biocatalyst, but there are clear differences between them. The most stable biocatalyst is that blocked with Cys, followed by the biocatalyst blocked using Gly (both clearly more stable than the enzyme immobilized on glutaraldehyde at pH 8), while the biocatalyst blocked with EDA and TrisEAE gave the lowest stabilities among these biocatalysts. That way, also the stability of the different biocatalysts differed, for both glutaraldehyde and VS-octyl ones, confirming that the enzyme presented very different functional features, our initial objective. Next, we have submitted the different biocatalysts to the incubation with metal phosphates.

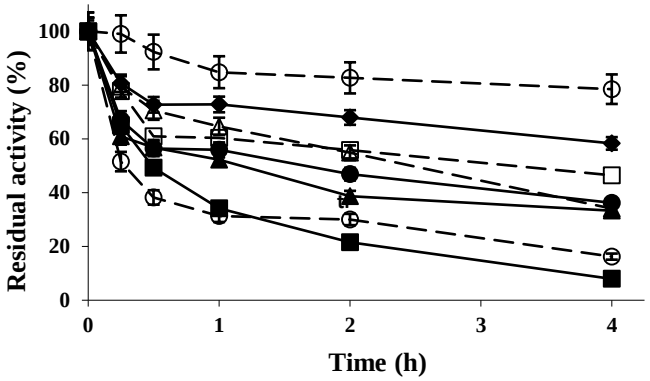

Figure 6.3. Inactivation courses of different TLL biocatalysts in 10 mM Tris-HCl buffer at pH 7.0 and 75 °C. Other specifications are described in Methods. TLL-octyl (solid square and solid line); Gly-TLL-VS-octyl (solid rhombus and solid line); EDA-TLL-VS-ocyl (solid circles and solid line); TrisAEA-TLL-VS-octyl (solid triangles and solid line); Asp-TLL-VS-octyl (open squares and dotted line); Cys-TLL-VS-octyl (open rhombus and dotted line); TLL-Glu (pH 5.0) (open circles and dotted line); TLL-Glu (pH 8.0) (open triangles and dotted line).

6.3.1.2 Effect of the treatment with metal phosphate of the different biocatalyst on enzyme activity

The biocatalysts were incubated with the three metal phosphates. Figure A6.4 shows how the biocatalysts were colored depending on the salt, while all biocatalysts presented a similar color when submitted to the same mineralization protocol. Exceptions are the glutaraldehyde biocatalysts, where the initial brown colors make color identification difficult, except for copper.

Table 6.2 shows the effect of the treatment with the different metal phosphates in the enzyme activity versus the different substrates.

Using the octyl preparation, the activity decreased using *p*-NPB as substrate by 25% using zinc, and by more than 40% using copper or cobalt. The activity versus triacetin increased by around 50% using zinc, while it almost remained unaltered using copper and cobalt. Using the methyl mandelates as substrates, the zinc salt promoted a 30% decrease for both isomers, using copper the activity versus the *R*-isomer remains at 85%, while versus the *S*-isomer it decreased below 60%. Using cobalt, the situation is the opposite, versus the *S*-isomer the activity decreased to 70%, while the activity versus the *R*- decreased to 60%.

Table 6.2. Effect of the treatment with metallic salt/phosphate on the mass activity (immobilized biocatalyst) of the different biocatalyst. The hydrolytic activity was measured using 1 mM *p*-NPB, 50 mM triacetin, and 50 mM *R*- or *S*-methyl mandelate at pH 7.0 and 25 °C. Experiments were performed as described in Methods.

Biocatalysts	Activity (U/g)															
	Without modification				Modification using $ZnCl_2$/sodium phosphate				Modification using $CuCl_2$/sodium phosphate				Modification using $CoCl_2$/sodium phosphate			
	***p*-NPB**	**Triacetin**	***R*-Mandelate**	***S*-Mandelate**	***p*-NPB**	**Triacetin**	***R*-Mandelate**	***S*-Mandelate**	***p*-NPB**	**Triacetin**	***R*-Mandelate**	***S*-Mandelate**	***p*-NPB**	**Triacetin**	***R*-Mandelate**	***S*-Mandelate**
TLL-octyl	1390.9 ± 25.0	110.9 ± 5.4	1.3 ± 0.06	0.7 ± 0.03	1069.43 ± 30.3	160.7 ± 7.2	0.9 ± 0.04	0.5 ± 0.02	797.6 ± 27.6	118.4 ± 4.8	1.1 ± 0.05	0.4 ± 0.02	784.0 ± 10.2	121.9 ± 5.1	0.8 ± 0.04	0.5 ± 0.02
Gly-TLL-VS-octyl	3279.0 ± 20.6	102.1 ± 4.1	1.2 ± 0.05	0.4 ± 0.02	3569.2 ± 125.2	119.3 ± 5.9	0.8 ± 0.03	0.2 ± 0.01	2411.7 ± 25.0	96.9 ± 3.6	0.8 ± 0.03	0.4 ± 0.02	3483.9 ± 12.1	119.7 ± 3.3	0.8 ± 0.03	0.3 ± 0.01
EDA-TLL-VS-octyl	3038.8 ± 51.7	83.6 ± 3.7	1.4 ± 0.02	0.3 ± 0.01	3505.6 ± 207.6	90.6 ± 3.1	0.7 ± 0.01	0.3 ± 0.01	3356.9 ± 86.8	130.6 ± 5.5	0.6 ± 0.03	0.3 ± 0.01	3902.8 ± 36.4	95.4 ± 4.8	0.8 ± 0.03	0.2 ± 0.01
TrisAEA-TLL-VS-octyl	3000.6 ± 28.3	94.0 ± 4.9	0.6 ± 0.02	0.07 ± 0.003	3540.2 ± 96.9	92.2 ± 4.7	0.7 ± 0.03	0.3 ± 0.01	2665.9 ± 159.1	64.8 ± 3.2	0.5 ± 0.02	0.4 ± 0.01	3611.2 ± 73.9	105.8 ± 4.1	0.5 ± 0.02	0.1 ± 0.004
Asp-TLL-VS-octyl	3109.6 ± 132.4	94.0 ± 3.7	0.5 ± 0.02	0.4 ± 0.02	3466.4 ± 141.8	143.4 ± 7.1	0.4 ± 0.01	0.4 ± 0.02	1934.9 ± 31.5	108.6 ± 5.4	0.2 ± 0.01	0.4 ± 0.02	3152.4 ± 170.4	126.0 ± 6.8	0.9 ± 0.04	0.4 ± 0.02
Cys-TLL-VS-octyl	2755.4 ± 98.1	64.1 ± 2.2	0.5 ± 0.02	0.2 ± 0.01	3470.4 ± 21.4	102.3 ± 5.4	0.4 ± 0.01	0.3 ± 0.02	2380.6 ± 85.6	90.9 ± 4.7	0.9 ± 0.04	0.4 ± 0.02	2006.0 ± 75.9	61.0 ± 3.0	0.8 ± 0.04	0.3 ± 0.01
TLL-Glu (pH 5.0)	37.6 ± 2.2	2.7 ± 0.1	10.6 ± 0.5	10.6 ± 0.5	32.4 ± 1.2	2.8 ± 0.1	5.4 ± 0.3	8.2 ± 0.4	57.4 ± 0.8	4.7 ± 0.2	2.4 ± 0.1	4.4 ± 0.2	21.1 ± 1.1	2.7 ± 0.1	3.4 ± 0.2	5.8 ± 0.3
TLL-Glu (pH 8.0)	15.6 ± 0.1	2.0 ± 0.1	9.1 ± 0.4	13.8 ± 0.7	24.0 ± 1.5	2.2 ± 0.1	8.9 ± 0.4	4.9 ± 0.2	25.0 ± 0.9	2.3 ± 0.1	0.9 ± 0.04	4.2 ± 0.2	18.0 ± 0.3	3.0 ± 0.1	1.0 ± 0.05	4.6 ± 0.2

Using the VS biocatalyst blocked with Gly, the treatment with zinc salts promoted the activity versus *p*-NPB to increase by less than 10%, versus triacetin by almost 20%, while the activity decreased versus both methyl mandelates. This effect of the zinc phosphate on enzyme activity and specificity is fully different to that found using octyl biocatalysts, where the activities decreased versus all substrates except triacetin. Using copper salts, the activity significantly decreased versus *p*-NPB (by 23%) and *R*-methyl mandelate (by one third), remaining similar versus triacetin and *S*-methyl mandelate. The treatment with cobalt salts produced a slight increase on the activity versus *p*-NPB (by more than 6%), increasing almost by 20% versus triacetin, with a similar decrease in the enzyme activity versus both isomers of methyl mandelate. That way, the response to the modification with the different salts of Gly-TLL-VS-octyl-agarose is very different to those found using the octyl preparation. This could be due to the enzyme distortion caused by the covalent immobilization or the interactions of the enzyme with the support.

Next, we studied the effect of the metal modification of the EDA-TLL-VS-octyl biocatalysts activities. The treatment with zinc increased the activity versus *p*-NPB by 15%, that way the zinc phospate- EDA-TLL-VS-octyl activity versus this substrate became similar to that of Zn phospate-Gly-TLL-VS-octyl. This treatment produced an increase of the activity versus triacetin (lower than 10%) and *R*-methyl mandelate (50%), while the activity versus *S*-methyl mandelate was maintained. When copper salts were used, the activity versus *p*-NPB increased by 10% and versus triacetin by 50% (becoming more active than TLL-octyl after any modification), and it remained unaltered for *S*-methyl mandelate while it decreased by more than 50% for the *R*-isomer. Cobalt salts produced the highest increase in activity of this biocatalyst versus *p*-NPB (by almost 30%, becoming the most active biocatalyst versus this substrate), the activity versus triacetin increased by 15%, and the activities versus both methyl mandelate isomers decreased. Again, this was different to the effect of the mineralization using the octyl-agarose, but also using the Gly-TLL-VS-octyl. That means that the enzyme-support interactions played an important role on the modulation of the enzyme mineralization effects.

The effects of the modification of TrisEAE-TLL-VS-octyl biocatalyst offers a different picture. Zinc phosphate treatment again increased the activity versus *p*-NPB (by 18%), maintaining the activities versus triacetin and *R*-methyl mandelate, while decreasing the activity by *S*-methyl mandelate (by almost 50%). The copper treatment reduced the activity versus *p*-NPB

(by more than 10%), versus triacetin (by more than 30%) and versus *R*- (by more than 15%) and *S*- (by more than 40%) methyl mandelate. Finally, the treatment with cobalt salt produced the highest increase in the activity versus *p*-NPB for this biocatalyst (over 20%) and it also increased the activity versus triacetin (by 12%), while it decreased the activity versus both methyl mandelate isomers, especially versus the *S*-isomer (maintaining only 14% of the initial activity). In all cases, the treatment made that the biocatalyst recovered the preference by the *R*-isomer, in the case of the treatment with cobalt, the activities ratio become 5.

Using Asp as blocking reagent, the treatment with zinc salts permitted to increase the activity versus *p*-NPB (by more than 11%), and very significantly with triacetin (by 50%, becoming the most active biocatalyst versus this substrate). The activity versus the methyl mandelate isomers was almost maintained. Using copper phosphate, the activity versus *p*-NPB decreased by almost 40%, versus triacetin it increased by more than 15%, versus *S*-methyl mandelate it was maintained, but versus the *R*-isomer it decreased to 40%. That way, this biocatalyst hydrolyzes the *S*-isomer twice as quickly as the *R*-isomer. The modification using the cobalt salt produced a small increase in the activity versus *p*-NPB, but the activity versus triacetin increased by almost 35%. The activities versus *R*-methyl mandelate also increased, by 1.8-fold, while versus the *S*-isomer remained unaltered.

Also, the modification of Cys-TLL-VS-octyl with the different salts offers a new different situation. Using zinc salts, the activity increased by more than 25% versus *p*-NPB, almost by 60% using triacetin, and decreased the activity versus *R*-methyl mandelate (by 20%) and increased the activity versus the *S*-isomer (by 40%). Using copper salts, the activity decreased versus *p*-NPB (by less than 15%), increased by more than 40% versus triacetin, 1.8-fold versus *R*-methyl mandelate and 2-fold versus the *S*-isomer. Using cobalt salts, the activity decreased almost by 30% versus *p*-NPB, 5% versus triacetin, but increased by 60% using *R*-methyl mandelate and 50% using the *S*-isomer.

That way, the effect of the different mineralizations on the enzyme activity and specificities strongly depend on the blocking reagent. Considering that the enzyme orientation should be identical for all biocatalysts and also the number of enzyme-support linkages [96], this suggested that the enzyme-support interactions may be very relevant for the final effects of the mineralization, without discarding a role of the groups in the support in the metal phosphate

crystal growth. Moreover, the metal phosphate treatment is able to tune the specificities of all the analyzed biocatalyst, each treatment favored some substrates while it is negative for others.

Next, we have analyzed the effect of the mineralization on the activity of both glutaraldehyde biocatalysts (Table 6.2). Here, the support surface is the same for both biocatalysts, but the enzyme orientation of the enzyme and the number of enzyme support bonds may be different (therefore, also the enzyme conformation) [82,85].

The modification with zinc salt of the biocatalyst immobilized at pH 5 produced a slight decrease on enzyme activity (by almost 15%) while the biocatalyst prepared at pH 8 increased the activity by 50%. Using triacetin as substrate, zinc mineralization did not present a relevant effect on enzyme activity, for the enzyme immobilized at pH 5 it increased by more than 3%, while for the enzyme immobilized at pH 8 it increased by 10%. Using the mandelate esters, the enzyme immobilized at pH 5 decreased the activity by almost 50% for the *R*-isomer, and by 27% for the *S*-isomer. The enzyme immobilized at pH 8 almost maintained the activity versus the *R*-isomer (from 9.1 U/g to 8.9 U/g), but it significantly decreased its activity versus the *S*-isomer (to just over 35%). The modification with copper salt promoted a significant increase of the activity versus *p*-NPB of the enzyme immobilized at pH 5 (by more than 50%) or at pH 8 (by 60%), also the activity versus triacetin increased (by almost 75% for the enzyme immobilized at pH 5 and by 15% for the enzyme immobilized at pH 8). The activity versus *R*-methyl mandelate was strongly reduced, to 22% using the enzyme immobilized at pH 5 and to 45% for the enzyme immobilized at pH 8, and versus the *S*-isomer the results were also negative: the activity decreased to 40% for the enzyme immobilized at pH 5 and to 30% using the enzyme immobilized at pH 8. The modification with cobalt salts produced a decrease in the activity versus *p*-NPB of the enzyme immobilized at pH 5 (to 56%) while it produced an increase for the enzyme immobilized at pH 8 (almost by 20%). The activity versus triacetin of the enzyme immobilized at pH 5 remained unaltered, while the enzyme immobilized at pH 8 increased its activity versus this substrate by 50% (becoming higher than the counterpart immobilized at pH 5). The activity versus *R*-methyl mandelate decreased due to this treatment, to 32% for the enzyme immobilized at pH 5 and to 11% for the enzyme immobilized at pH 8. Furthermore, the activity versus the *S*-isomer decreased by this mineralization, to less than 55% for the enzyme immobilized at pH 5 and to around 33% for the other biocatalyst.

Again, the effects of the mineralization strongly depended on the used biocatalysts. In this instance, the support surface is exactly the same, but the orientation and the number of enzyme linkages may be different. However, in all cases the mineralization permits to alter the enzyme specificity, increasing or decreasing the activity versus some substrates depending on the metal and the immobilization protocol.

Combining salts and immobilization protocol, it seems that the potential of TLL mineralization to tune the enzyme features may become really impressive.

6.3.1.3 Effect of the mineralization on enzyme stability

Figure 6.4 shows the inactivation courses of the differently biocatalysts of TLL-octyl-agarose. As it has been previously reported [70], the stability of the enzyme strongly improved after modification with Zn^{2+}, while Cu^{2+} even promoted a slight destabilization and Co^{2+} treatment almost did not present any significant effect.

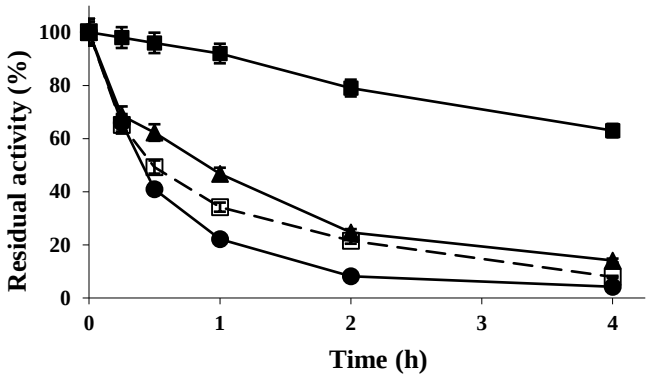

Figure 6.4. Inactivation courses of differently mineralized TLL-octyl biocatalysts in 10 mM Tris-HCl buffer at pH 7.0 and 75 °C. Other specifications are described in Methods. Unmodified TLL-octyl (open squares and dotted line); TLL-octyl modified with $ZnCl_2$/sodium phosphate (solid squares); $CuCl_2$/sodium phosphate (solid circles); $CoCl_2$/sodium phosphate (solid triangles).

Using Gly-TLL-VS-octyl (Figure 6.5a), Zn^{2+} treatment is also the most stabilizing treatment, however, the stabilization obtained using Co^{2+} is more significant than using the octyl biocatalyst, while Cu^{2+} maintained its destabilizing effect. Moving to EDA-TLL-VS-octyl (Figure 6.5b), the results show that all salt treatments had no significant effects on enzyme

stability. For TrisEAE-TLL-VS-octyl (Figure 6.5c), all modifications presented a destabilizing effect, only relevant for Cu^{2+} that became a very destabilizing treatment for this enzyme formulation. Asp-TLL-VS-octyl (Figure 6.5d) offered a new picture; now Co^{2+} and Zn^{2+} mineralization exhibited a strong stabilizing effect, very similar, while Cu^{2+} showed a small destabilizing effect. The situation is similar for Cys-TLL-VS-octyl (Figure 6.5e), although in this case the destabilizing effect of Cu^{2+} mineralization is clearer. That way, as it occurred with the activity effects of the mineralization, the effects on enzyme stability of this enzyme modification strategy strongly depend on the blocking reagent (which alters the enzyme structure [96]).

The inactivation courses of the glutaraldehyde preparations are showed in Figure 6.6. For the enzyme immobilized at pH 5 (Figure 6.6a), Zn^{2+} and Co^{2+} treatment produced a strong stabilization, while Cu^{2+} produced a strong destabilization. If the enzyme is immobilized at pH 8 (Figure 6.6b), Co^{2+} is the only mineralization that clearly improved the enzyme stability (in a lower extension that for the enzyme immobilized at pH 5), Zn^{2+} almost did not alter enzyme stability, while Cu^{2+} dramatically destabilized the enzyme. This confirmed that the enzyme orientation or number of enzyme-support bonds may be critical to determine the effect of the mineralization on the enzyme stability.

6.3.2 The case of CRL biocatalysts

6.3.2.1 Immobilization of CRL on the different supports

Figure A6.5 shows the immobilization course of CRL on octyl-agarose. Around 80% of the enzyme activity became immobilized in 2 h. The immobilization of the enzyme on octyl-VS maintained the immobilization yield in 80% (Figure A6.6), and also the immobilization yield was very similar on glutaraldehyde at pH 5 and 8 (Figure A6.7).

Table 6.3 shows the activities of the different biocatalysts versus the 4 model substrates. CRL-octyl had the highest activity versus *p*-NPB, more than 11-fold higher than versus triacetin. That activity was less than 5-fold lower than the activity versus *R*-methyl mandelate. The activities ratio with both isomers was almost 2.8.

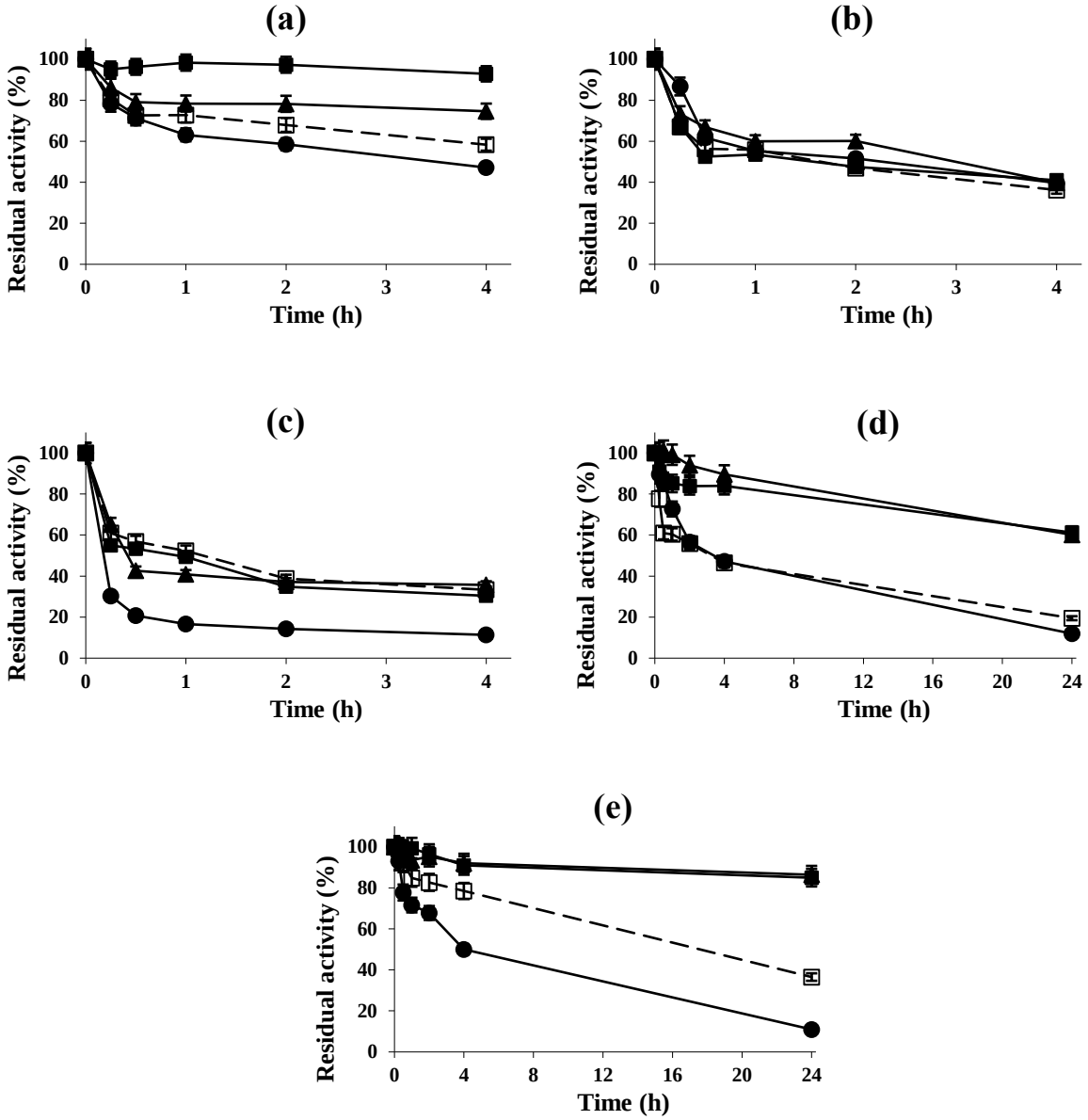

Figure 6.5. Inactivation course of different TLL-VS-octyl biocatalysts modified with metallic salt/sodium phosphate: (a) Gly-TLL-VS-octyl, (b) EDA-TLL-VS-octyl, (c) TrisAEA-TLL-VS-octyl, (d) Asp-TLL-VS-octyl, (e) Cys-TLL-VS-octyl. The biocatalysts were inactivated at 75 °C, in presence of 10 mM Tris-HCl buffer at pH 7.0. Other specifications are described in Methods. Unmodified biocatalyst (open squares and dotted line); biocatalyst modified with $ZnCl_2$/sodium phosphate (solid squares); $CuCl_2$/sodium phosphate (solid circles); $CoCl_2$/sodium phosphate (solid triangles).

Table 6.3. Mass activity (immobilized biocatalyst) of different CRL biocatalysts versus different substrates: 50 mM *R*- or *S*-methyl mandelate (pH 7 and 25 °C), 50 mM of triacetin (pH 7 and 25 °C) and 1 mM of *p*-NPB (pH 7 and 25 °C). Experiments were performed as described in Methods.

Biocatalysts	**Activity (U/g)**			
	***p*-NPB**	**Triacetin**	***R*-Mandelate**	***S*-Mandelate**
CRL-octyl	614.3 ± 36.7	49.3 ± 2.4	10.8 ± 0.5	3.9 ± 0.9
Gly-CRL-VS-octyl	1761.0 ± 58.6	0.7 ± 0.03	9.2 ± 0.5	3.4 ± 0.2
EDA-CRL-VS-octyl	1461.5 ± 28.3	0.5 ± 0.03	4.9 ± 0.2	3.4 ± 0.2
TrisAEA-CRL-VS-octyl	995.8 ± 14.5	0.3 ± 0.01	4.8 ± 0.2	4.0 ± 0.2
Asp-CRL-VS-octyl	1875.6 ± 91.68	0.7 ± 0.03	6.9 ± 0.3	4.1 ± 0.2
Cys-CRL-VS-octyl	1129.9 ± 49.7	0.3 ± 0.01	7.2 ± 0.2	4.4 ± 0.1
CRL-Glu (pH 5.0)	844.4 ± 25.69	36.8 ± 1.8	8.1 ± 0.5	8.8 ± 0.4
CRL-Glu (pH 8.0)	825.9 ± 10.8	27.1 ± 1.4	9.6 ± 0.5	11.1 ± 0.5

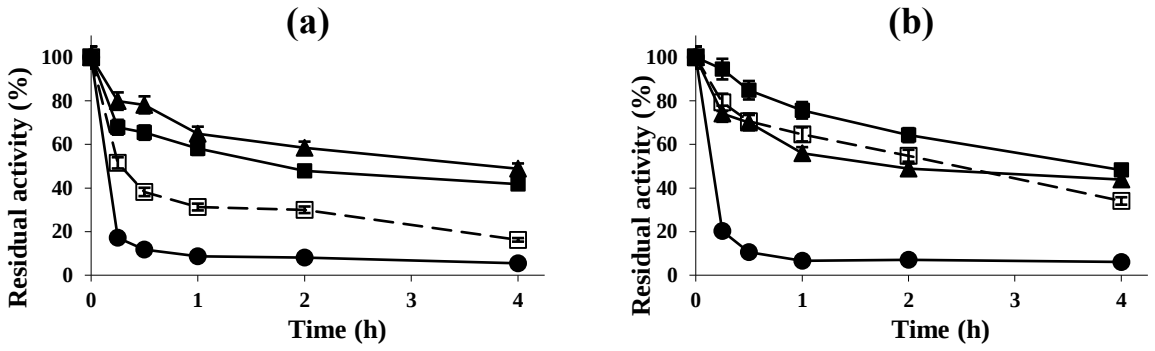

Figure 6.6. Inactivation courses of differently mineralized TLL-Glu biocatalysts immobilized at (a) pH 5.0 and (b) pH 8.0. The biocatalysts were inactivated at 75 °C, in presence of 10 mM Tris-HCl buffer at pH 7.0. Other specifications are described in Methods. Unmodified TLL-Glu (open squares and dotted line); TLL-Glu modified with $ZnCl_2$/sodium phosphate (solid squares); $CuCl_2$/sodium phosphate (solid circles); $CoCl_2$/sodium phosphate (solid triangles).

The use of Gly-CRL-octyl biocatalyst permitted to greatly increase the activity versus *p*-NPB (by almost 2.9), while it promoted a drop in the activity versus triacetin (from more than 49 U/g to 0.7), slightly reducing the activities versus both isomers of methyl mandelate. Blocking the biocatalyst with EDA gave 2.4 higher *p*-NPB activity than the octyl biocatalyst, lowering the activity versus triacetin to 1% and for *R*-methyl mandelate to 45% and to 87% for the *S*-isomer. The use of TrisAEA gave an increase on the *p*-NPB activity of 1.6 compared with the octyl preparation. The activity with triacetin was of only 0.3 U/g. Using *R*-methyl mandelate the activity decreased to less than 45%, while with the *S*-isomer the activity marginally increased. Blocking the biocatalyst with Asp produced a biocatalyst 3-fold more active than the octyl biocatalyst using *p*-NPB, it reduced the activity versus *R*-methyl mandelate (to 64%) while it slightly increased the activity versus the *S*-isomer (by a 5%). Again, the activity versus triacetin was strongly affected, maintaining only 0.7 U/g. The blocking with Cys gave an increase in activity versus *p*-NPB of 1.8-fold. The activity versus triacetin suffered a drop, maintaining only 0.3 U/g, while the activity versus *R*-methyl mandelate decreased by one third and the activity versus the *S*-isomer increased by more than 10%. That way, the activities of the different biocatalyst prepared in octyl (VS) support were greatly different. Explanations for these results may be as described above for TLL. Again, we have built biocatalysts having different functional properties.

Focusing on the glutaraldehyde biocatalysts, the activity versus *p*-NPB was not very different for both biocatalysts, and it was 30-40% higher than using CRL-octyl. The activity versus triacetin was higher for the biocatalyst immobilized at pH 5 than at pH 8 (35% higher), but lower than the enzyme immobilized on octyl agarose (by more than 25%). Both glutaraldehyde biocatalysts hydrolyzed the *S*- methyl mandelate more rapidly than the *R*-methyl mandelate. The enzyme immobilized at pH 5 was less active with both isomers (around 80%) than the octyl preparation. However, the enzyme immobilized at pH 8 was less active versus the *R*-isomer than the octyl preparation (by more than 10%), and more active versus the *S*-isomer (2.8 fold). That way, enzyme specificity and enantiospecificity were very different for the different biocatalysts. Again, our objective has been accomplished.

Next, the enzyme stability of the different CRL biocatalysts was compared. Figure 6.7 shows the inactivation courses of the different CRL-biocatalysts. CRL-octyl is the least stable biocatalyst, being the enzyme immobilized on glutaraldehyde at pH 8 less stable than the enzyme

immobilized at pH 5, even though at pH 8 a higher number of enzyme-supports bonds may be expected. This suggests that the immobilization pH may alter the enzyme orientation on the support, as enzyme orientation also alters the effects of the immobilization on enzyme stability [84–87,89,90,92–94,113], and the immobilization achieved at pH 5 seems to involve more relevant areas for the enzyme stability [114,115]. The VS-octyl biocatalysts stabilities are in between the glutaraldehyde and the octyl biocatalyst, without too large differences (the blocking with TrisAEA produced the least stable biocatalysts, while the blocking with Gly gave the most stable preparation).

That way, again the functional properties of the different biocatalysts were quite different, and these biocatalysts were used for mineralization using diverse salts.

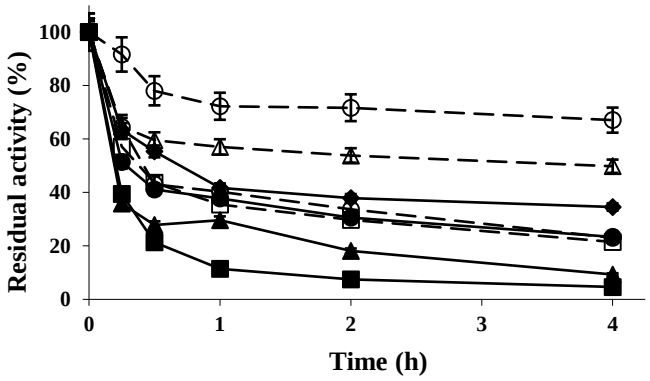

Figure 6.7. Inactivation courses of different CRL biocatalysts in 10 mM Tris-HCl buffer at pH 7.0 and 70 °C. Other specifications are described in Methods. CRL-octyl (solid square and solid line); Gly-CRL-VS-octyl (solid rhombus and solid line); EDA-CRL-VS-ocyl (solid circles and solid line); TrisAEA-CRL-VS-octyl (solid triangles and solid line); Asp-CRL-VS-octyl (open squares and dotted line); Cys-CRL-VS-octyl (open rhombus and dotted line); CRL-Glu (pH 5.0) (open circles and dotted line); CRL-Glu (pH 8.0) (open triangles and dotted line).

6.3.2.2 Effect of the treatment with metal phosphate of the different biocatalyst on enzyme activity

The different biocatalysts were treated with the different salts, Figure A6.8 shows that the colors were similar to those observed using TLL. Table 6.4 offers the effect of the mineralization of the biocatalyst on their activities versus the different substrates.

Starting with CRL-octyl, the use of zinc salts produced an increase of its *p*-NPB activity by around 10%, it decreased the activity versus triacetin by 30% and versus both methyl mandelate isomers by around 10%. Using copper salts, the increase on the activity with *p*-NPB is almost 15%, activities versus triacetin and *R*-methyl mandelate are fairly maintained and the activity versus *S*-methyl mandelate decreased by almost 20%. Cobalt phosphate mineralization increased the activity versus *p*-NPB (by less than 5%) and decreased the activity versus triacetin (to 83%), *R*- (to 88%) and *S*- (to 75%) methyl mandelate.

The situation is different using Gly-CRL-VS-octyl, the modification with zinc phosphate had marginal effects on the enzyme activity, slightly decreased the activity versus *p*-NPB and *S*-methyl mandelate (to 95%), it maintained the activity versus triacetin, and increased the activity versus *R*-methyl mandelate (by 5%). The use of copper salts also produced marginal effects on the biocatalysts features, while cobalt mineralization increased the activity versus *p*-NPB (by 12%), *R*- and *S*-methyl mandelate (by more than 7%), while the activity versus triacetin remained unaltered. Similar small increases or decreases in activities could be detected for the other CRL-VS-octyl biocatalysts, never exceeding 15%. It seemed that the mineralization presented a much smaller effect for these CRL biocatalysts than when using TLL.

The changes on enzyme activity induced by the mineralization using the glutaraldehyde biocatalysts were clearer. The enzyme immobilized at pH 5 slightly decreased its *p*-NPB activity after zinc mineralization, while the enzyme immobilized at pH 8 slightly increased its activity. Using triacetin, the enzyme immobilized at pH 5 increased its activity by 33%; if the enzyme was immobilized at pH 8, the increase on the activity is by 65%. If *R*-methyl mandelate was used as substrate, the activity of the enzyme immobilized at pH 5 marginally increased, while the enzyme immobilized at pH 8 decreased its activity by more than 5%. Using the *S*-isomer, the enzyme immobilized at pH 5 increased the activity after this mineralization by 8%, while if the immobilization was performed at pH 8, the activity decreased to less than 80%.

Table 6.4. Effect of the treatment with metallic salt/phosphate on the activity of the different CRL biocatalyst versus different substrates. The hydrolytic activity was measured using 1 mM *p*-NPB, 50 mM triacetin, and 50 mM *R*- or *S*-methyl mandelate at pH 7.0 and 25 °C. Experiments were performed as described in methods.

Biocatalysts	Activity (U/g)															
	Without modification				Modification using $ZnCl_2$/sodium phosphate				Modification using $CuCl_2$/sodium phosphate				Modification using $CoCl_2$/sodium phosphate			
	p-NPB	Triacetin	*R*-Mandelate	*S*-Mandelate	*p*-NPB	Triacetin	*R*-Mandelate	*S*-Mandelate	*p*-NPB	Triacetin	*R*-Mandelate	*S*-Mandelate	*p*-NPB	Triacetin	*R*-Mandelate	*S*-Mandelate
CRL-octyl	614.3 ± 36.7	49.3 ± 2.4	10.8 ± 0.5	3.9 ± 0.9	675.7 ± 29.4	34.5 ± 1.7	9.6 ± 0.5	3.5 ± 0.1	701.6 ± 25.1	51.1 ± 2.6	9.9 ± 0.5	3.2 ± 0.2	639.4 ± 23.2	40.89 ± 2.1	9.5 ± 0.5	2.9 ± 0.1
Gly-CRL-VS-octyl	1761.0 ± 58.6	0.7 ± 0.03	9.2 ± 0.5	3.4 ± 0.2	1674.7 ± 48.2	0.7 ± 0.03	9.7 ± 0.5	3.6 ± 0.2	1628.4 ± 8.08	0.5 ± 0.02	9.1 ± 0.4	3.3 ± 0.1	1974.1 ± 10.5	0.7 ± 0.02	9.9 ± 0.5	3.6 ± 0.2
EDA-CRL-VS-octyl	1461.5 ± 28.3	0.5 ± 0.03	4.9 ± 0.2	3.4 ± 0.2	1461.1 ± 12.92	0.5 ± 0.03	4.7 ± 0.4	3.5 ± 0.2	1276.73 ± 21.8	0.5 ± 0.02	4.5 ± 0.3	3.6 ± 0.2	1630.8 ± 9.69	0.5 ± 0.02	5.1 ± 0.4	3.9 ± 0.2
TrisAEA-CRL-VS-octyl	995.8 ± 14.5	0.3 ± 0.01	4.8 ± 0.2	4.0 ± 0.2	1067.5 ± 40.9	0.4 ± 0.01	4.6 ± 0.3	4.0 ± 0.3	1012.5 ± 29.48	0.4 ± 0.02	4.8 ± 0.2	4.0 ± 0.2	941.86 ± 30.9	0.3 ± 0.01	5.0 ± 0.3	4.1 ± 0.2
Asp-CRL-VS-octyl	1875.6 ± 91.68	0.7 ± 0.03	6.9 ± 0.3	4.1 ± 0.2	1721.7 ± 46.1	0.8 ± 0.03	7.5 ± 0.4	4.0 ± 0.2	1860.2 ± 56.5	0.8 ± 0.03	7.0 ± 0.4	3.9 ± 0.2	1813.9 ± 70.3	0.8 ± 0.03	7.0 ± 0.4	4.1 ± 0.2
Cys-CRL-VS-octyl	1129.9 ± 49.7	0.3 ± 0.01	7.2 ± 0.2	4.4 ± 0.1	1153.1 ± 43.2	0.4 ± 0.01	6.9 ± 0.3	4.8 ± 0.2	994.32 ± 63.8	0.3 ± 0.01	7.5 ± 0.4	4.3 ± 0.1	1122.2 ± 70.9	0.4 ± 0.02	7.4 ± 0.3	4.5 ± 0.3
CRL-Glu (pH 5.0)	844.4 ± 25.69	36.8 ± 1.8	8.1 ± 0.5	8.8 ± 0.4	835.7 ± 35.7	48.5 ± 2.4	8.3 ± 0.3	9.5 ± 0.5	777.4 ± 23.8	17.2 ± 0.9	7.8 ± 0.3	7.6 ± 0.4	853.2 ± 20.2	46.9 ± 2.3	8.5 ± 0.5	9.4 ± 0.5
CRL-Glu (pH 8.0)	825.9 ± 10.8	27.1 ± 1.4	9.6 ± 0.5	11.1 ± 0.5	849.1 ± 31.5	44.9 ± 2.3	8.1 ± 0.4	8.8 ± 0.4	550.5 ± 23.2	18.8 ± 0.9	8.0 ± 0.4	7.8 ± 0.4	701.6 ± 16.3	44.9 ± 2.2	7.8 ± 0.4	7.9 ± 0.4

Analyzing the effects of the copper salts treatment, the activity versus *p*-NPB decreased almost by 10% for the enzyme immobilized at pH 5, while the enzyme immobilized at pH 8 lost one third of the activity. Using triacetin as substrate, the enzyme immobilized at pH 5 decreased the activity to less than 50%, the other biocatalyst only lost 10% of the activity. Both biocatalysts decreased their activities versus both methyl mandelate, but more for the *S*- than for the *R*-isomer. The activities ratios became inverted after the treatment, now the immobilized and copper mineralized enzyme preferred the *R*-isomer.

Finally, using cobalt salts, the activity versus *p*-NPB marginally increased for the enzyme immobilized at pH 5, the increase is more significant for its triacetin activity (by 27%). For *R*- and *S*-methyl mandelate, the increase in activity is small (5 and 7%). The enzyme immobilized at pH 8 decreased its *p*-NPB activity to 85%, while it increased its triacetin activity by 1.65-fold. Using methyl mandelate, the activity decreased for both isomers, more for the *S*-isomer.

That way, although not as significant as in the case of TLL, the combination of immobilization protocol and mineralization salt permitted to tune the specificity and activity of immobilized CRL.

6.3.2.3 Effect of the mineralization on enzyme stability

Next, the effect of the mineralization on the biocatalyst's stability was analyzed. Using CRL-octyl (Figure 6.8), all mineralized biocatalysts were more stable than the unmodified octyl, being the biocatalyst modified with cobalt salts less stabilized than using the other salts and that biocatalyst modified with zinc salts was the most stable. The situation was fairly different using Gly-CRL-VS-octyl (Figure 6.9a), where the copper salt produced an enzyme destabilization and the stabilization obtained using zinc phosphate is much smaller than using the octyl biocatalyst. For EDA-CRL-VS-octyl (Figure 6.9b), the treatments had a moderate effect on enzyme stability, slightly negative, being more destabilizing the mineralization using copper. TrisAEA-CRL-VS-octyl (Figure 6.9c) stability was not affected by the treatments, except using copper, which had a negative effect. Asp-CRL-VS-octyl (Figure 6.9d) and Cys-CRL-VS-octyl (Figure 6.9e) was slightly stabilized using cobalt and zinc salts, and marginally destabilized using copper salts.

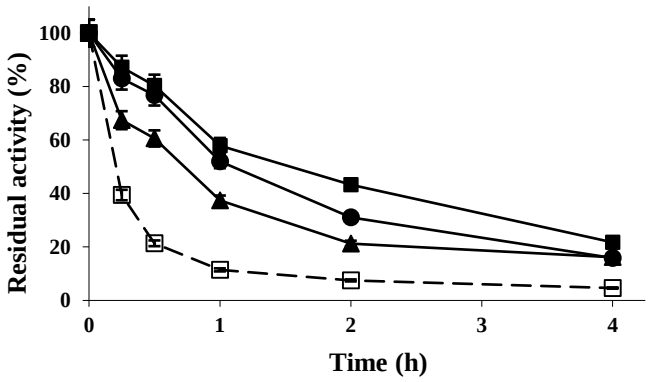

Figure 6.8. Inactivation courses of differently mineralized CRL-octyl biocatalysts in 10 mM Tris-HCl buffer at pH 7.0 and 70 °C. Other specifications are described in Methods. Unmodified CRL-octyl (open squares and dotted line); CRL-octyl modified with $ZnCl_2$/sodium phosphate (solid squares); $CuCl_2$/sodium phosphate (solid circles); $CoCl_2$/sodium phosphate (solid triangles).

Moving to the glutaraldehyde biocatalyst, the differences caused by the immobilization pH are very clear. While the stability of the enzyme immobilized at pH 5 (Figure 6.10a) was almost not affected by the mineralization using cobalt and zinc salts, copper produced a drastic destabilization. Using the enzyme immobilized at pH 8 (Figure 6.10b), the zinc salt produced a clear stabilization, followed by the cobalt salt, while the copper mineralization produced an enzyme destabilization, although less relevant than using the enzyme immobilized at pH 5.

That way, the mineralization effect on enzyme stability on immobilized CRL strongly depended on the immobilization protocol. In some instances, a mineralization protocol produced a great stabilization, while in others the same protocol even produced a negative effect. Using CRL, the effects may be less significant than using TLL, but they are still very relevant.

6.3.3 Operational stability of the biocatalysts in hydrolysis of methyl mandelic esters

Although the objective of this paper was not to optimize any reaction, all the biocatalysts were reused 3 times in hydrolysis cycles of methyl mandelate hydrolysis (both *R*- and *S*-isomers). The activities of all biocatalysts were maintained during the 3 cycles over 95% (nor shown results), as expected from the high stability of the octyl-lipase and glutaraldehyde-lipase

preparations, even the destabilized biocatalysts maintained their activities under these mild conditions. Moreover, the color induced by the metal was maintained also almost intact after these 3 reuses.

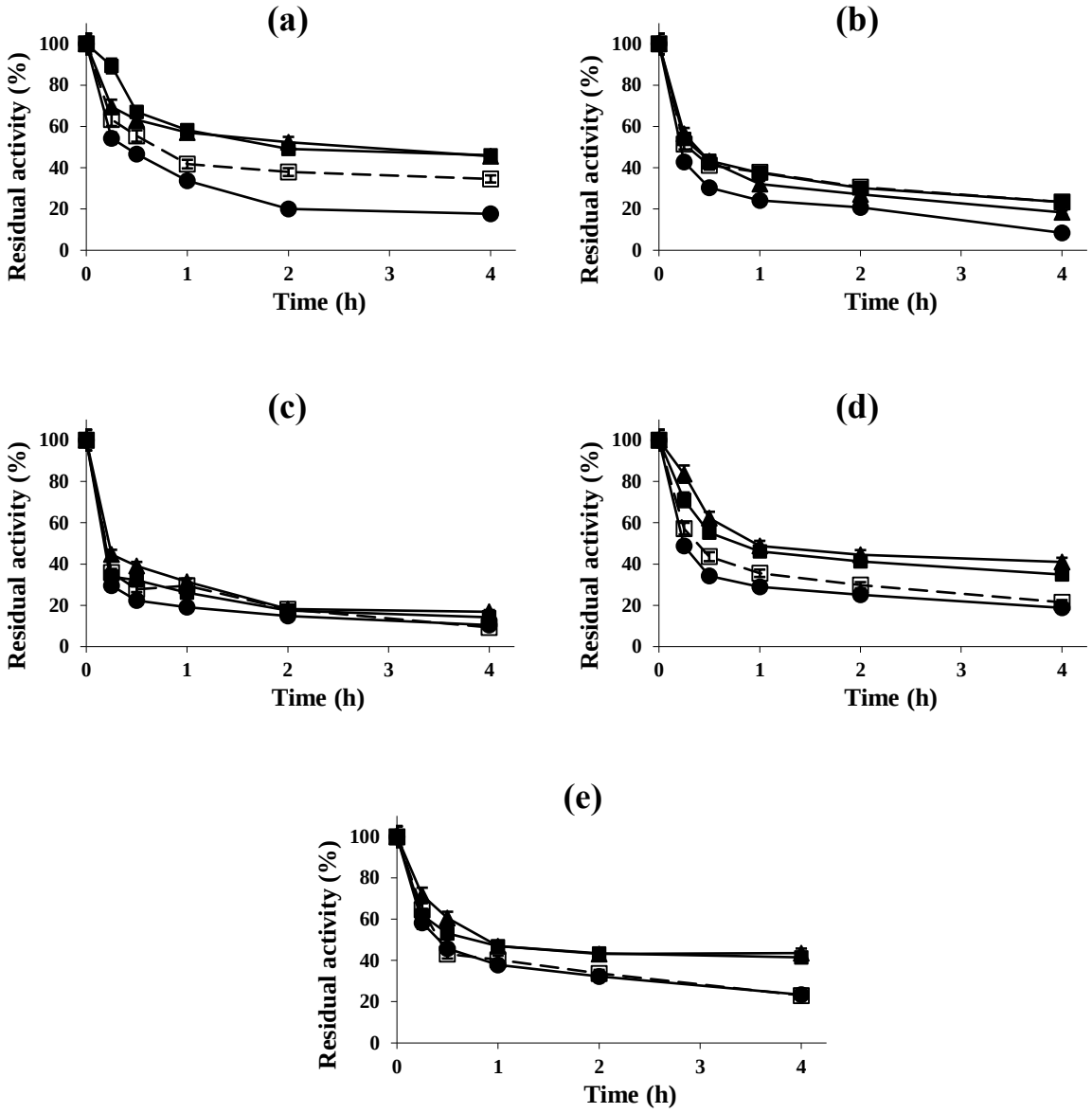

Figure 6.9. Inactivation course of different CRL-VS-octyl biocatalysts modified with different metallic salt/sodium phosphate: (a) Gly-CRL-VS-octyl, (b) EDA-CRL-VS-octyl, (c) TrisAEA-CRL-VS-octyl, (d) Asp-CRL-VS-octyl, (e) Cys-CRL-VS-octyl. The biocatalysts were inactivated at 70 °C, in presence of 10 mM Tris-HCl buffer at pH 7.0. Other specifications are described in Methods. Unmodified biocatalyst (open squares and dotted line); biocatalyst modified with

$ZnCl_2$/sodium phosphate (solid squares); $CuCl_2$/sodium phosphate (solid circles); $CoCl_2$/sodium phosphate (solid triangles).

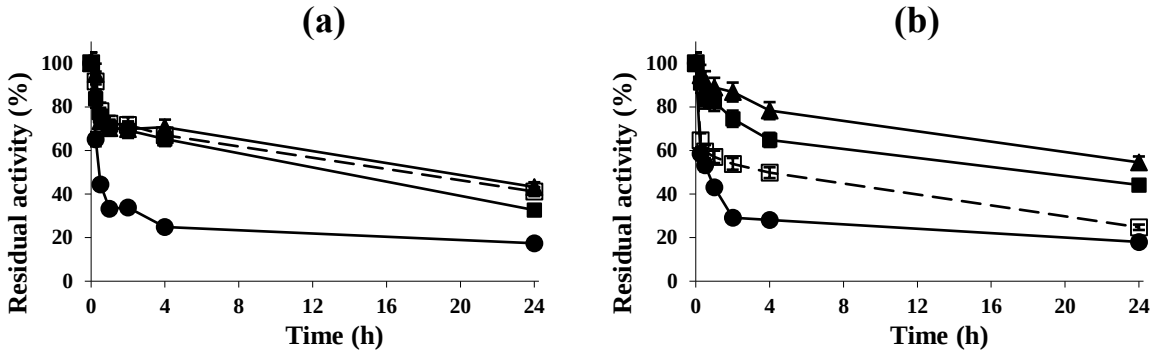

Figure 6.10. Inactivation courses of CRL-Glu biocatalysts immobilized at (a) pH 5.0 and (b) pH 8.0. The biocatalysts were inactivated at 70 °C, in presence of 10 mM Tris-HCl buffer at pH 7.0. Other specifications are described in Methods. Unmodified CRL-Glu (open squares and dotted line); CRL-Glu modified with $ZnCl_2$/sodium phosphate (solid squares); $CuCl_2$/sodium phosphate (solid circles); $CoCl_2$/sodium phosphate (solid triangles).

6.4 Conclusion

The results shown in this paper are new examples on the power of enzyme immobilization to tune enzyme functional features, including examples where the only difference is the final physical interactions between enzyme and support (i.e., octyl-vinyl sulfone biocatalysts blocked with different reagents). Now, we have clearly shown how the immobilization protocol determines the effect on enzyme functional properties of enzyme mineralization using metal phosphates. This occurs even if the only difference between the different biocatalysts is the groups located surface of the support, and therefore, the enzyme-support interactions. Changes on enzyme stability or activity versus a specific substrate caused by the mineralization using a specific metal salt may be positive or negative depending on the immobilization protocol. In the used examples, TLL seems to be more responsible to these changes than CRL, but in both cases the differences are significant enough. This means that to select the best immobilization protocol/enzyme/mineralization protocol, the researcher should analyze the final enzyme features in the specific process where the enzyme will be utilized (substrate, reaction conditions).

This simple strategy opens the door to integrate enzyme immobilization and enzyme mineralization to increase the library of biocatalysts from a single lipase that can be available to optimize each specific process. Moreover, this suggests that the results obtained by mineralization of a specific immobilized biocatalyst cannot be directly extrapolated to other biocatalysts. The use a wide battery of metals and immobilization protocols can permit to "create biocatalysts" adequate for many processes, even if the original enzyme does not look very adequate for the specific process [47,116].

It could be highly interesting to investigate the different structural changes that the enzyme can adopt as consequence of its interaction with the different support surfaces, as this can give some clues of the reasons for the different activities and stabilities of the biocatalysts [96]. The potential of biophysical, physic-chemical and spectroscopic techniques to advance in the structural analysis of immobilized enzymes and understand the structure-function relationship of the enzymes is advancing very rapidly and their potential (today still they have some limitations) [117,118], to improve the understanding the phenomena that determine the final properties of the immobilized enzyme may open new opportunities in the development more controllable and efficient immobilization process [117–126]. Although the future of these techniques gives us a reason to be optimistic, the current scenario is they are still far from can purpose a clear finger-printing that can explain the functional features of an immobilized enzymes.

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APPENDIX

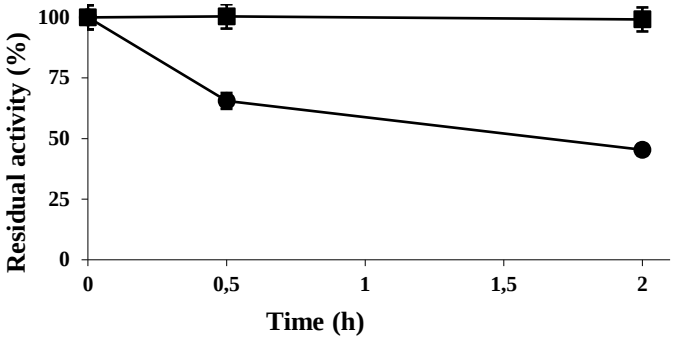

Figure A6.1. Immobilization course of TLL on octyl agarose using an enzyme loading of 20 mg/g. The immobilization was performed in 5 mM sodium phosphate at 25 °C and pH 7.0. Squares: reference and circles: supernatant. Other specifications are described in Methods.

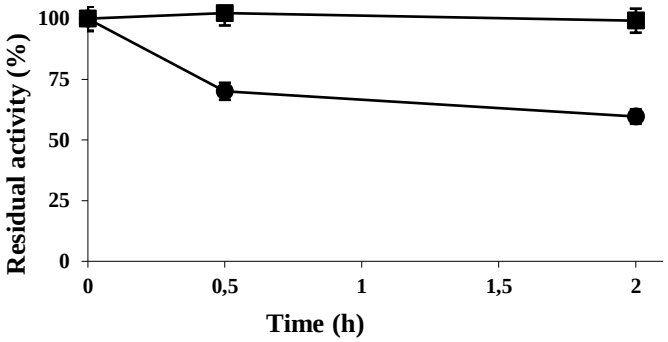

Figure A6.2. Immobilization course by interfacial activation of TLL on octyl-vinyl sulfone agarose beads using an enzyme loading of 20 mg/g. The immobilization was performed in 5 mM sodium acetate at 25 °C and pH 5.0. Squares: reference and circles: supernatant. Other specifications are described in Methods.

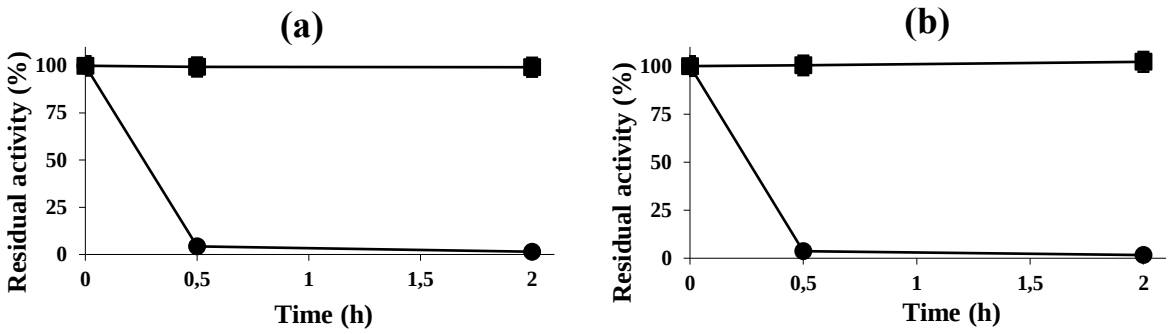

Figure A6.3. Immobilization course of TLL on amino-glutaraldehyde-agarose beads using an enzyme loading of 20 mg/g at (a) pH 5.0 and (b) pH 8.0. Squares: reference and circles: supernatant. Other specifications are described in Methods.

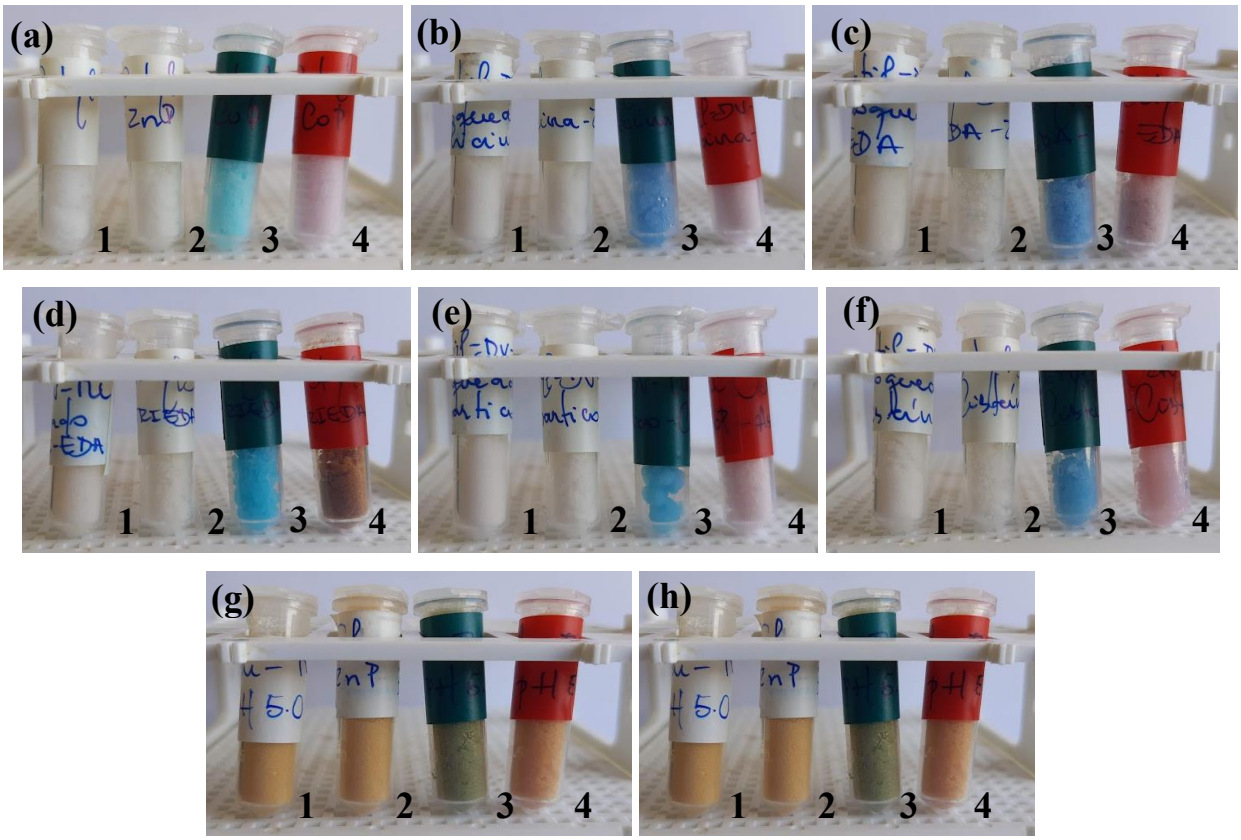

Figure A6.4. Photo of the different TLL biocatalysts. (a) TLL-octyl, (b) Gly-TLL-VS-ocyl, (c) EDA-TLL-VS-ocyl, (d) TrisAEA-TLL-VS-ocyl, (e) Asp-TLL-VS-ocyl, (f) Cys-TLL-VS-ocyl, (g) TLL-Glu (pH 5.0), and (h) TLL-Glu (pH 8.0) (1) unmodified and (2-4) modified with (2) $ZnCl_2$/sodium phosphate, (3) $CuCl_2$/sodium phosphate and (4) $CoCl_2$/sodium phosphate.

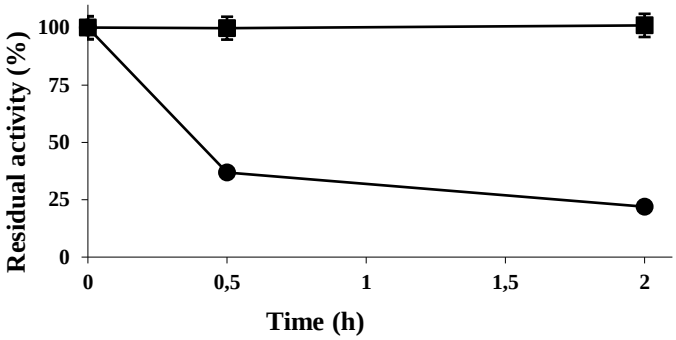

Figure A6.5. Immobilization course of CRL on octyl agarose using an enzyme loading of 30 mg/g. The immobilization was performed in 5 mM sodium phosphate at 25 °C and pH 7.0. Squares: reference and circles: supernatant. Other specifications are described in Methods.

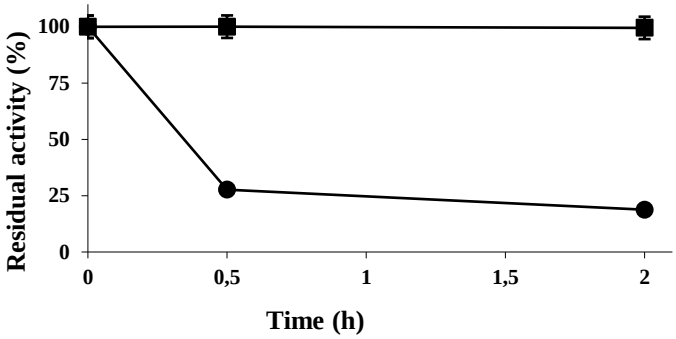

Figure A6.6. Immobilization course by interfacial activation of CRL on octyl-vinyl sulfone agarose beads using an enzyme loading of 30 mg/g. The immobilization was performed in 5 mM sodium acetate at 25 °C and pH 5.0. Squares: reference and circles: supernatant. Other specifications are described in Methods.

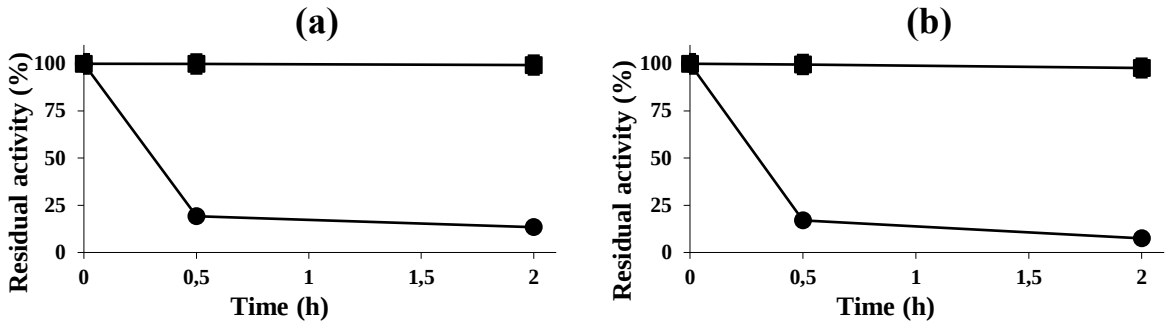

Figure A6.7. Immobilization course of CRL on amino-glutaraldehyde-agarose beads using an enzyme loading of 30 mg/g at (a) pH 5.0 and (b) pH 8.0. Squares: reference and circles: supernatant. Other specifications are described in Methods.

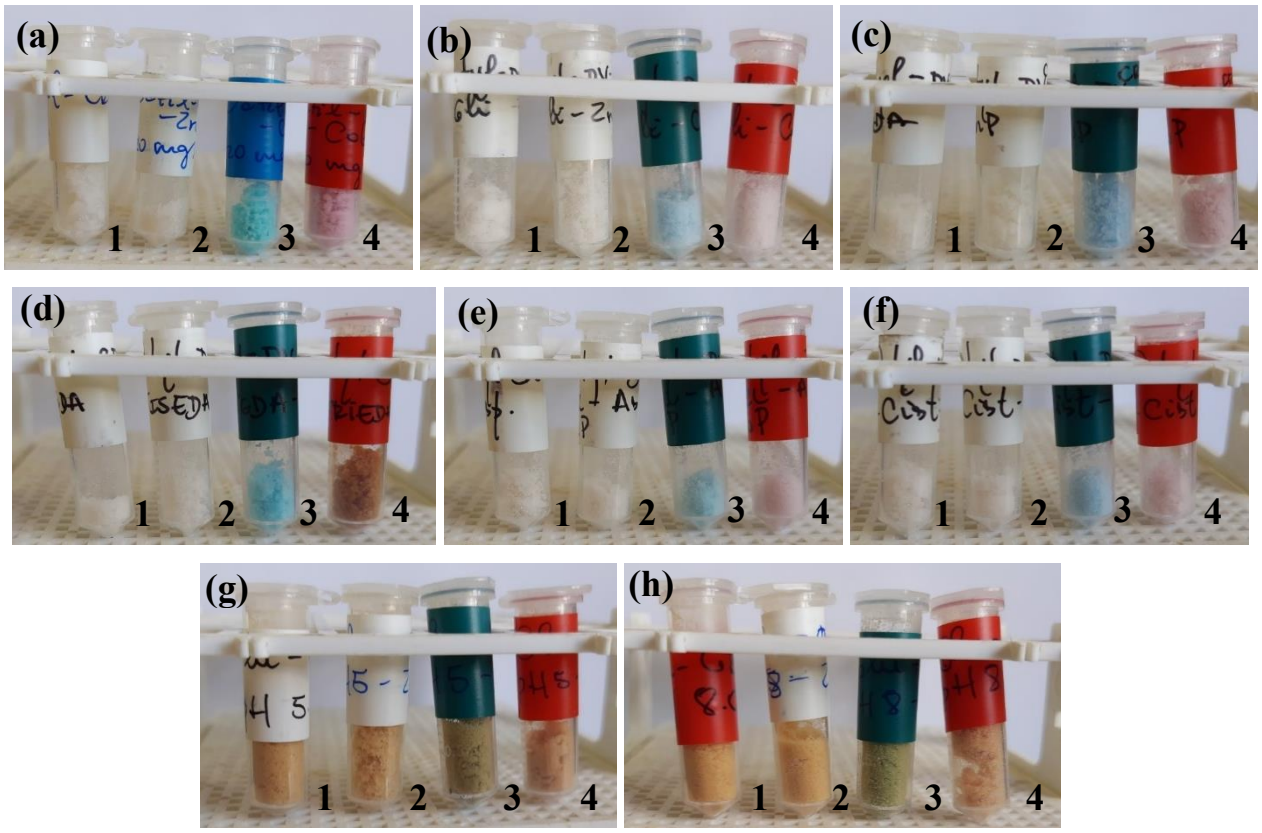

Figure A6.8. Photo of different CRL biocatalysts CRL-octyl, (b) Gly-CRL-VS-ocyl, (c) EDA-CRL-VS-ocyl, (d) TrisAEA-CRL-VS-ocyl, (e) Asp-CRL-VS-ocyl, (f) Cys-CRL-VS-ocyl, (g) CRL-Glu (pH 5.0), and (h) CRL-Glu (pH 8.0) (1) unmodified and (2-4) modified with (2) $ZnCl_2$/sodium phosphate, (3) $CuCl_2$/sodium phosphate and (4) $CoCl_2$/sodium phosphate.

CHAPTER 7
TUNING IMMOBILIZED ENZYME FEATURES BY COMBINING SOLID-PHASE PHYSICOCHEMICAL MODIFICATION AND MINERALIZATION

Lipase B from *Candida antarctica* (CALB) and lipase from *Thermomyces lanuginosus* (TLL) have been immobilized on octyl agarose. Then, the biocatalysts have been chemically modified using glutaraldehyde, trinitrobenzenesulfonic acid or ethylenediamine and carbodiimide, or physically coated with ionic polymers, such as polyethylenimine (PEI) and dextran sulfate. These produced alterations of the enzyme activities have in most cases negative effects with some substrates and positive with other ones (e.g., amination of immobilized TLL increased the activity versus p-nitro phenyl butyrate *(p*-NPB), reduced the activity with *R*-methyl mandate by half and maintained the activity with *S*-isomer). The modification with PEI increased 8-fold the biocatalyst activity versus *R*-methyl mandelate. Enzyme stability was also modified, usually improving (e.g., the modification of immobilized TLL with PEI or glutaraldehyde enabled to maintain more than 70% of the initial activity when the unmodified enzyme maintained less than 50%). The immobilized enzymes were also mineralized by using phosphate metals (Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} or Mg^{2+}), and this affected also enzyme activity, specificity (e.g., immobilized TLL increased its activity after zinc mineralization versus triacetin while its activity increased versus all the other assayed substrates) and stability (e.g., the same modification increase the residual stability from almost 0 to more than 60%) , depending on the enzyme a metal could be positively, neutrally or negatively affected for a specific feature. Finally, we analyzed if the chemical modification could, somehow, tune the effects of the mineralization. Effectively, the same mineralization could have very different effects on the same immobilized enzyme if it has been previously submitted to different physicochemical modifications. The same mineralization could present different effects on the enzyme activity, specificity, or stability, depending on the previous modification performed on the enzyme, showing these previous enzyme modifications alter the effects of the mineralization on enzyme features. For example, TLL modified with glutaraldehyde and treated with zinc salts increased its activity using *R*-methyl mandelate, while almost maintaining its activity versus the other substrates unaltered, while the aminated TLL maintained its activity with both methyl mandelate isomers while it decreased with *p*-NPB and triacetin. TLL was found to be easier to tune than CALB by the strategies used in this paper. That way, the combination of chemical or physical modifications of enzyme previously to their mineralization

increase the range of features that the immobilized enzyme can exhibit, enabling to enlarge the biocatalyst library.

7.1 Introduction

Enzymes are attracting increasing attention as industrial catalysts due to their high activity under mild conditions, high substrate specificity and product selectivity [1–3], and the huge evolution of the strategies to solve their limitations imposed by their biological origin. Metagenomics enable to utilize the whole biodiversity (making enzyme from non-cultivable or even disappeared organisms available) [4–6], genetic tools enable to tailor the desired feature (using enzyme modelling and site-directed mutagenesis [7,8] or directed evolution [9–11]), physicochemical enzyme modifications are more efficient and controlled each passing day. This enables a battery of solutions to overcome any enzyme deficiency [12,13]. Moreover, some of these solutions may be used in a conjoined way, for example making the building of enzymes bearing several active centers feasible [14], such as plurizymes [15–17].

In this context, enzyme immobilization may be remarked. It was initially developed as solution to the problems raised by enzyme solubility, the preparation of heterogeneous biocatalysts made enzyme recovery and reuse simpler (if the enzyme was still active) [18–20]. Nowadays, many researchers have shown that immobilization may solve many enzyme limitations. One of the most pursued objectives is enzyme stabilization, that may be accomplished by diverse reasons [21,22]. The fact that the enzyme is distorted when immobilized and is in a confined space (using porous supports) also produces an alteration of enzyme selectivity, specificity, activity, response to inhibitors, etc. [23–25], that can greatly increase the reactions where the enzyme can be utilized with satisfactory results. Moreover, the enzyme may be purified during the immobilization process [26,27].

An especially interesting possibility is the simultaneous use of immobilization as a way to simplify other enzyme improvement strategies. That way, solid-phase chemical modification of enzymes is much simpler than the modification of the free enzyme. It also prevents undesired enzyme aggregations, and if the enzyme is stabilized by the immobilization, it may be also more resistant to changes in the physical features of the enzyme surface caused by the chemical modification [28–31]. Recently, it has been proposed also that the mineralization of enzymes in solid phase may have some advantages [32,33]. The building of enzyme hybrid nanoflowers (where a salt crystal grows around the enzyme molecules that act

as nucleation points) as an immobilization system have proved to allow the improvement of enzyme stability and activity in certain cases [34–43], but the small size and fragility of the resulting structures makes their recovery complex. Some researchers propose the use of magnetic materials or the trapping of these nanoflowers in larger and mechanically more stable structures as a solution for these limitations [44–51]. The mineralization of previously immobilized lipases has been recently proposed as a possibility to obtain some benefits from mineralization but with mechanically stable biocatalyst [32,33]. It has been showed that the use of fully loaded biocatalyst permits to maximize the effects, and that the crystals also grown in the support pores in absence of enzymes with similar intensity [32]. Moreover, it has been shown that the enzyme orientation and/or enzyme conformation can greatly alter the final effects of this mineralization on enzyme features [52]. In this new communication, we evaluate if the enzyme chemical modification of immobilized enzymes can somehow alter the results observed by the enzyme mineralization [52]. It has been shown in many papers how the chemical or physical modification of immobilized enzymes may have different effects depending on the enzyme immobilization protocol [31,53–56]. The enzyme mineralization is really a physical modification of the enzyme, and it can be expected that the effects may depend on the enzyme exact conformation and orientation concerning the support. In fact, it has been shown that some ions may be very negative or positive for immobilized lipase stability, but only when the enzymes were immobilized following certain specific protocol [57,58].

This may depend on the exact chemical composition of the protein external surface that will be the subject of the modification. Different chemical compositions may lead to different degrees of enzyme coating by the metal crystals. In fact, recently, researchers have shown how protein folding and molecular modification define the rate, extent, and mechanism of crystallization when mixed with 2-methylimidazole and zinc acetate [59]. We can assume that these effects may be also produced when mineralizing immobilized enzymes, and that they can produce different effects on the enzyme properties.

Lipases have been used as model enzymes in this study, as they are among the most used enzymes at both academic and industrial levels [60–63]. They have a peculiar mechanism of action, called interfacial activation, as they have two possible conformations (closed and open), that in homogeneous media are in equilibrium [64–67]. A polypeptide called lid is able to isolate the active center in most lipases. In the presence of any hydrophobic surface (a drop of substrate, a hydrophobic protein, but also other open form of the lipase or a hydrophobic support), the lipase becomes strongly adsorbed with the lid shift leaving the active center exposed [64–67]. This has caused lipase immobilization on hydrophobic supports to become a

very popular immobilization strategy, enabling the one step immobilization, purification, stabilization and hyperactivation of the lipases [27]. This has been the immobilization strategy utilized in this paper, using octyl-agarose beads as support [68]. Then, the immobilized enzymes have been submitted to diverse physicochemical modifications to analyze if this can tune the effects of the mineralization. Moreover, we can observe if some of the modified groups are necessary to get the mineralization effects, or not. The first modification was that using glutaraldehyde [69,70]. The utilized modification conditions with glutaraldehyde ensures the full modification of all primary amino groups in the enzyme surface with just one molecule of glutaraldehyde [71], making the enzyme surface slightly more hydrophobic (glutaraldehyde is mildly hydrophobic) but leaving the ionization capability to the amino group. This can stabilize the enzyme due to the promotion of inter- (making enzyme release more difficult) [72] and intramolecular (increasing enzyme rigidity) crosslinkings [73]. The amination of the enzyme with ethylenediamine (EDA) and carbodiimide permits to transform all external carboxylic groups in amino groups, altering the ionic interactions (now, all will be repulsion interactions) [31,74,75]. Finally, the modification of the enzyme with picrylsulfonic acid or trinitrobenzenesulfonic acid (TNBS) is highly selective for the primary amino groups [76], and makes that the ionization capability of the amino group disappeared, as it becomes an amide, promoting the enzyme surface hydrophobization. Finally, the enzyme surface has been coated using polyethyleneimine (PEI) [77], or dextran sulfate [78,79], both polymers will almost fully coat the enzyme surface and permit the intermolecular crosslinking, making enzyme release from the support difficult [80]. The polymers offer an open structure permitting the entry of small compounds to the enzyme surface but generating some partition effects due to their ionic character. In this study, we have selected the lipases from *Thermomyces lanuginosus* [81], and *Candida antarctica* (form B) [82–84], that are among the most popular ones, and examples where both, the solid phase mineralization [32,33] and the chemical modification presented interesting results in previous publications [55,56,72,85–89]. That way, the objectives of this paper can be summarized in an effort to analyze whether the coupled solid phase chemical or physical modification of enzymes and their further mineralization may have additive effects on the enzyme features, influencing from the first to the results achieved by the second.

7.2 Materials and Methods

7.2.1 Materials

A TLL liquid formulation with 20.77 mg protein/mL was utilized in this paper while lipase B from *Candida antarctica* (CALB) was a liquid formulation with 7.7 mg protein (kindly donated by Novozymes Spain (Madrid, Spain). Bradford´s reagent (utilized to calculate the protein concentration [90]), *p*-nitrophenyl-butyrate (*p*-NPB), triacetin, *R*- and *S*-methyl mandelate, acetonitrile for HPLC (gradient grade, ≥99.9%), glutaraldehyde (GA) solution (25% in H_2O), ethylenediamine (EDA), picrylsulfonic acid (TNBS), polyethylenimine (PEI, MW 25,000), dextran sulfate (DS, MW 20,000), N-3-(Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (ECD), $NiCl_2$, $MgCl_2$, $CoCl_2$, $CuCl_2$ and $ZnCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Octyl Sepharose® CL-4B was acquired from GE Healthcare (Uppsala, Sweden). All other reagents were of analytical grade.

7.2.2 Methods

All experiments were performed at least by triplicate, and the values are presented as mean values and standard deviation.

7.2.2.1 Immobilization of lipases on octyl-agarose beads

The lipases were immobilized by interfacial activation on octyl agarose beads using enzyme loads over the capacity of the support to ensure the full support surface coating (TLL: 20 mg/g and CALB: 25 mg/g) [91,92]. 1 g of support was added to 10 mL of enzyme solution prepared in 5 mM sodium phosphate at pH 7.0. The immobilization was conducted at room temperature under gentle stirring for 2 h. The enzyme activity in the supernatant, suspension and a reference were quantified using *p*-NPB assay throughout the immobilization course. Afterwards, the suspensions were vacuum filtered, washed 10 folds with 20 volumes of distilled water, and stored at 4-6 °C.

7.2.2.2 Immobilization of lipases on octyl-agarose beads

The immobilized enzymes were treated with 1% (v/v) GA, aminated using 2 M of EDA following the carbodiimide route, or modified with 1 mM TNBS, 10% (w/v), 10% (w/v) PEI or 10% (w/v) DS.

The treatment with GA was performed according to Wang et al. [93], adding 0.4 mL of GA solution (25% in H_2O) in 10 mL of immobilized enzyme solution (0.1 g/mL) prepared

in 5 mM phosphate buffer at pH 8.0. The modification was carried out at room temperature under gentle agitation for 1 h. At the end, the suspensions were vacuum filtered, washed 10 folds with 20 volumes of distilled water, and stored at 4-6 °C.

For surface amination of the immobilized enzyme, 1 g of immobilized enzyme was added to 10 ml of 2 M EDA at pH 4.75. Then, solid ECD was added to reach a concentration of 10 mM. The amination was carried out at room temperature for 2 h. Under these conditions, 100% modification of all the exposed carboxylic groups was achieved [74]. At the end, the suspensions were vacuum filtered, washed 10 folds with 20 volumes of dissolved water, and stored at 4-6 °C.

The enzyme surface modification with TNBS followed the methodology of Snyder and Sobocinski [76]. 58.8 μL of TNBS (1 mM final concentration) was added to 10 mL of solution containing the immobilized enzyme (0.1 g/mL) prepared in 5 mM sodium phosphate at pH 8.0. The modification was carried out at room temperature under gentle agitation for 3 h. At the end, the suspensions were vacuum filtered, washed 10 folds with 20 volumes of distilled water, and stored at 4-6 °C.

The modification with PEI or DS followed the methodology of Arana-Peña et al. [91] and Virgen-Ortíz et al. [94], respectively. 1 g of immobilized enzymes was treated with 10 mL of a solution 10% (w/v) of PEI or DS at pH 7.0. The modification was carried out at room temperature under gentle agitation for 18 h. Afterwards, the biocatalysts were vacuum filtered, washed 10 folds with 20 volumes of distilled water, and stored at 4-6 °C.

7.2.2.3 Modification of immobilized enzyme with metallic salt/phosphate

The immobilized enzymes were modified with metallic salt/phosphate following the methodology described by Guimarães et al. [32]. 400 μL of metallic salt solution were added to 5 mL immobilized enzymes solution (0.1 g/mL) prepared in 10 mM sodium phosphate/125 mM NaCl at pH 7.4. The enzyme treatment was conducted at room temperature under gentle stirring for 5 h. Afterwards, the biocatalysts were vacuum filtered, washed 10 folds with 20 volumes of distilled water, and stored at 4-6 °C.

7.2.2.4 Thermal inactivation of different lipase biocatalysts

In a standard experiment, 1 g of immobilized biocatalyst was suspended in 10 mL of 10 mM Tris-HCl at pH 7.0 and incubated at 68 °C or 75 °C. Periodically, samples of 50 μL of the inactivation suspensions were collected to determine their residual activities. Residual

activities were defined as current activity divided by the initial one in percentage. The experiments were performed employing *p*-NPB as substrate.

7.2.2.5 Determination of the biocatalysts activities versus different substrates

One unit of activity (U) was defined as the amount of enzyme that hydrolyzes one μmol of substrate per minute under the described conditions. Considering the strong tendency of lipases, and specifically TLL, to form lipase-lipase dimers by involving the open forms of two lipase molecules, the use of free enzymes could drive to complex results and we have preferred to focus on the comparison of the different immobilized enzymes [95–101]. Octyl agarose provide the monomeric and open form of the lipases preventing this kind of problems [27,68].

7.2.2.5.1 Hydrolysis of p-NPB

50 μL of 50 mM *p*-NPB prepared in acetonitrile was added to 2.5 mL of 25 mM sodium phosphate at pH 7.0 and the reaction was started by adding 50 μL of soluble or immobilized enzyme sample to this mixture. The reaction was conducted using a thermostatization system at 25 °C under magnetic stirring for 1.5 min. The *p*-nitrophenol released into the medium was monitored by spectrophotometry at 348 nm (isosbestic point) to determine the hydrolytic activity ($\varepsilon = 5150\ M^{-1}\ cm^{-1}$) [102].

7.2.2.5.2 Hydrolysis of triacetin

50 mg of immobilized enzyme were added to 3 mL of 50 mM of triacetin prepared in 50 mM of sodium phosphate at pH 7.0. The reaction was carried out at room temperature under gentle stirring. The quantification of hydrolysis degree was determined by the release of 1,2 and 1,3 diacetin (under these conditions, the 1,2 diacetin produced undergoes acyl migration giving 1,3 diacetin) in the reaction medium [103]. A Waters 486 chromatograph (Waters, Millford, USA) presenting a Kromasil C18 column (15 cm x 0.46 cm) and a UV/VIS detector (set to 230 nm) was employed in the analyses to determine the degree of conversion (two points over 5% and under 25%, to ensure linearity and minimize experimental error caused by the initial acid content of the samples) and enzymatic activity. The mobile phase composed 85% (v/v) water and 15% (v/v) acetonitrile with a flow rate of 1 mL/min. The retention times were 4 min for 1,2 and 1,3 diacetins (under these conditions. they eluted at the same retention time) and 18 min for triacetin [91].

7.2.2.5.3 Hydrolysis of R- or S-methyl mandelate

50 mg of immobilized lipase were added to 3 mL of 50 mM *R*- or *S*-methyl mandelate in 50 mM sodium phosphate solution at pH 7.0. The reaction was carried out at room temperature under gentle stirring. The quantification of hydrolysis was determined by the release of mandelic acid in the reaction medium. A Waters 486 chromatograph (Waters, Millford, USA) presenting a Kromasil C18 column (15 cm x 0.46 cm) and a UV/VIS detector (set to 230 nm) was employed in the analyses to determine the degree of conversion (two points over 5% and under 25%, to ensure linearity and minimize experimental error caused by the initial acid content of the samples) and enzymatic activity [104]. The mobile phase was 10 mM ammonium acetate and acetonitrile (65-35% (v/v)) at pH 2.8 with a flow rate of 1 mL/min. The retention times were 2.5 min for mandelic acid and 4.2 min for the *R*- or *S*-methyl mandelate [105]. Activities ratio was defined as the activity versus the *R*-isomer/activity versus the *S*-isomer.

7.3 Results and Discussion

7.3.1 Preparation of the immobilized and chemically modified TLL-Biocatalysts

TLL was immobilized on octyl agarose, as shown in Figure A7.1, around 50% of the enzyme activity is incorporated to the support after 2 h (time found in previous papers to be enough to fully coat the support surface with lipases under these conditions) [106–111]. Then, the biocatalyst was submitted to the different modifications described in introduction. Table 7.1 summarizes the effect of the modification on the activity versus different substrates. The highest activity was found using *p*-NPB assay, and the lowest one using the *R*-methyl mandelate, being the activity more than 2.5-fold higher using the *S*-isomer. The modification with glutaraldehyde marginally decreased the activity using the *p*-NPB assay, decreased its activity by 20% in the triacetin assay, by 1/3 using *R*-methyl mandelate and by 1/4 using *S*-methyl mandelate assays (increasing the activities ratio). The amination produced almost a 20% increase in the activity versus *p*-NPB, a 44% increase versus triacetin, while the activity versus *R*-methyl mandelate decreased to 50% and versus the *S*-isomer, it is maintained (doubling the activities ratio). The modification with TNBS increased the activity using *p*-NPB by a 25%. The activity was not affected when using triacetin and it decreased using *R*-methyl mandelate while it almost did not affect the activity using the *S*-isomer (again increase the activities ratio). That way, all these chemical modifications affected enzyme specificity and enantiospecificity, in many cases based on an increase in the activity using some of the activity assays. To explain the reasons for these

effects is complex, as it has been shown that the effect of the chemical or physical modification not only depends on the enzyme, but also on the immobilization protocol [31,53–56].

Table 7.1. Specific activity of different biocatalysts with 1 mM *p*-NPB (pH 7 and 25 °C), 50 mM triacetin (pH 7 and 25 °C), and 50 mM *R*- or *S*-methyl mandelate (pH 7 and 25 °C). Experiments were conducted as described in Methods.

Biocatalysts	**Activity (U/g)**			
	***p*-NPB**	**Triacetin**	***R*-Methyl Mandelate**	***S*-Methyl Mandelate**
Octyl-TLL	1420 ± 50	601 ± 6	1.2 ± 0.1	3.2 ± 0.2
Octyl-TLL-GA	1350 ± 90	48 ± 2	0.8 ± 0.1	2.4 ± 0.1
Octyl-TLL-Amin	1670 ± 50	88 ± 4	0.6 ± 0.1	3.2 ± 0.2
Octyl-TLL-TNBS	1760 ± 80	60± 3	0.9 ± 0.1	3.1 ± 0.2
Octyl-TLL-PEI	1360 ± 60	90 ± 4	9.8 ± 0.5	7.7 ± 0.4
Octyl-TLL-DS	980 ± 50	28 ± 1	7.5 ± 0.4	6.5 ± 0.2

The physical coating of the immobilized TLL with PEI produced a small decrease in the activity using the *p*-NPB assay, but the activity almost increased by 50% using triacetin. The most interesting result is the great increase of the immobilized enzyme activity versus both methyl mandelates, 8.2 folds using the *R*-isomer and 2.4 using the *S*-isomer, with a preference for the *R*-isomer from the enzyme. The modification using DS decreased the activity using *p*-NPB (to 70%) and triacetin (to 46%), while it significantly increased the activities versus the methyl mandelates, by 6.25 using the *R*-isomer and by just 2-fold using the *S*-isomer. Again, the modified enzyme prefers the *R*-isomer. That way, these modifications also strongly tuned the enzyme specificity, as previously described [55,56,72,85–89].

Next, the stabilities of the different biocatalysts had been evaluated (Figure 7.1). The modifications with glutaraldehyde and PEI produced significant stabilizations, while the modification with TNBS and DS almost did not affect enzyme stability, and the enzyme amination promoted a decrease in enzyme stability. It should be considered that the proximity of the enzyme molecules (we are using fully loaded biocatalysts) can affect enzyme stability, and that the chemical modifications will not only affect the intramolecular enzyme interactions or the possibility of intermolecular crosslinking, also enzyme molecule-enzyme molecule interactions may be altered [106,107,112]. In any case, it seems that the inter- and

intramolecular crosslinkings induced by glutaraldehyde can have positive effects on the immobilized enzyme stability (rigidifying the enzyme structure and hindering enzyme desorption from the support) [70,72,113] or the intermolecular crosslinking achieved by using PEI for the enzyme stability [78,80]. DS should produce similar crosslinking, but apparently the anionic environment generated by this molecule is not positive for enzyme stability.

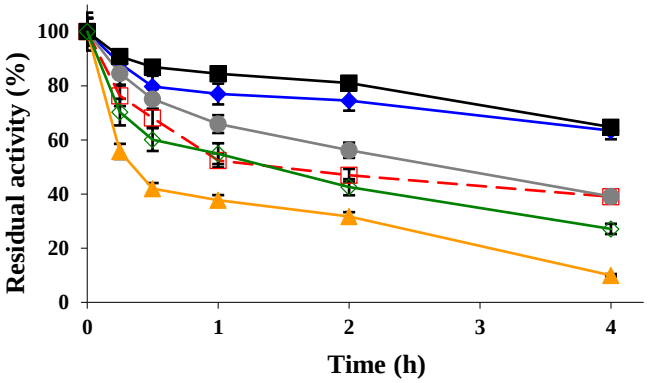

Figure 7.1. Inactivation courses of different physically and chemically modified octyl-TLL biocatalysts. The biocatalysts were inactivated at 68 °C, in presence of 10 mM Tris-HCl buffer at pH 7.0. Other specifications are described in Methods. Unmodified octyl-TLL (empty squares and dashed and red line); octyl-TLL modified with 1% glutaraldehyde (solid rhombus and solid blue line); amination using 2 M ethylenediamine (solid triangles and solid orange line); 1 mM picrylsulfonic acid (solid circles and solid grey line); 10% polyethylenimine (solid squares and solid black line); 10% dextran sulfate (empty rhombus and solid green line).

7.3.2 Modification of octyl-TLL with different phosphate salts

Next, the effects of the modification of octyl-TLL biocatalyst with Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} and Mg^{2+} on its activity and stability were studied. Table 7.2 summarizes the activity results. In the *p*-NPB assay, only the modification with Mg^{2+} produced a slight increase in enzyme activity (by more than 15%), being the less active biocatalysts those modified using Cu^{2+} (with an activity just over 80%). In the case of triacetin assay, the activity only decreased using Ni^{2+} (to around 88%), the other modifications slightly increased the activity in this assay by around 10%. Using *R*-methyl mandelate, Ni^{2+} and Mg^{2+} produced an increase of the enzyme activity (by 25%), Cu^{2+} treatment produce a drastic decrease in activity (by 1/3), while Zn^{2+} has a marginal effect. Using the *S*-isomer, all biocatalysts decreased their activity, being the enzyme treated with Mg^{2+} the least active one (75% activity compared to the untreated biocatalyst).

That way, the *R/S* activities ratio changes after the different mineralizations. This shows how this mineralization is also able to tune the immobilized enzyme specificity; it is not possible to state a mineralization as universally positive or negative for octyl-TLL activity, as that depends on the substrate. These effects should be the results of conformational changes induced by the mineralization, changes that yield a more active enzyme form for some substrates while for other substrates the effects are negative.

The stabilities of the different biocatalysts were then analyzed (Figure 7.2). The mineralization with Zn^{2+} produced the highest stabilization, Ni^{2+} also produced a relevant stabilization, Co^{2+} treatment produced a lower stabilization, and Cu^{2+} treatment promoted a slight destabilization. Mg^{2+} mineralization had no effect on enzyme stability.

Next, we have compared these mineralization effects using the enzymes previously modified.

Table 7.2. Specific activity of different TLL biocatalysts with 1 mM *p*-NPB (pH 7 and 25 °C), 50 mM triacetin (pH 7 and 25 °C), and 50 mM *R*- or *S*-methyl mandelate (pH 7 and 25 °C). Experiments were conducted as described in Methods.

Biocatalysts	**Activity (U/g)**			
	***p*-NPB**	**Triacetin**	***R*-Methyl Mandelate**	***S*-Methyl Mandelate**
Octyl-TLL	1420. ± 50	61 ± 6	1.2 ± 0.1	3.2 ± 0.2
Octyl-TLL-ZnP*	1040 ± 60	88 ± 6	0.9 ± 0.1	3.0 ± 0.1
Octyl-TLL-CoP	1210 ± 70	65 ± 5	1.1 ± 0.1	2.9 ± 0.2
Octyl-TLL-CuP	1140 ± 90	67 ± 6	0.8 ± 0.1	2.7 ± 0.1
Octyl-TLL-NiP	1370 ± 90	53 ± 3	1.5 ± 0.1	3.0 ± 0.2
Octyl-TLL-MgP	1660 ± 40	66 ± 3	1.5 ± 0.1	2.4 ± 0.1

* ZnP, CuP, CoP, NiP, MgP correspond to the metallic salt/sodium phosphate.

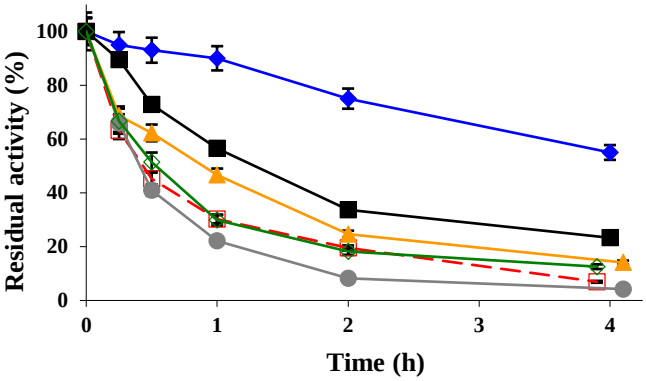

Figure 7.2. Inactivation courses of different octyl-TLL biocatalysts in 10 mM Tris-HCl buffer at pH 7.0 and 75 °C. Other specifications are described in Methods. Unmodified octyl-TLL (empty squares and dashed and red line); octyl-TLL modified with 1% glutaraldehyde (solid rhombus and solid blue line); amination using 2 M ethylenediamine (solid triangles and solid orange line); 1 mM picrylsulfonic acid (solid circles and solid grey line); 10% polyethylenimine (solid squares and solid black line); 10% dextran sulfate (empty rhombus and solid green line).

7.3.3 Effect of the mineralization on the immobilized enzyme previously modified

Starting with the enzyme modified with glutaraldehyde, Figure A7.2 shows that the color acquired by the glutaraldehyde modified biocatalysts was fairly similar to that observed using the unmodified biocatalyst for all metals. The mineralization produced a slight increase in *p*-NPB activity using Zn^{2+} and Mg^{2+}, Ni^{2+} has almost no effect while Co^{2+} and Cu^{2+} produced a decrease by over 15% (Table 7.3, Line 2-7). Using triacetin, activity is almost unaltered using Zn^{2+}, Mg^{2+} and Ni^{2+}, while it decreased using Co^{2+} and Cu^{2+} (by almost 30%). All mineralization produced a decrease in the activity versus *R*-methyl mandelate, not very relevant except using Co^{2+} (the activity becomes 25%), using the *S*-isomer the picture is quite different, as Zn^{2+} treatment increased the activity by 25%, Mg^{2+} maintained the activity, and Ni^{2+}, Cu^{2+} and Co^{2+} produced the highest decrease in activity (from 2,4 to 2.2, 2.1 and 2.0 respectively) (Table 7.3, Line 2-7). That way, enzyme activities and specificity were altered, although in a different form to the mineralization of the unmodified biocatalyst. The changes in activity should be related to alterations in the enzymes' conformation, which are positive for some substrates and negative for others.

Table 7.3. Specific activity of different immobilized and chemically modified TLL biocatalysts after mineralization. The hydrolytic activity was measured using 1 mM *p*-NPB (pH 7 and 25 °C), 50 mM triacetin (pH 7 and 25 °C), and 50 mM *R*- or *S*-methyl mandelate (pH 7 and 25 °C). Experiments were conducted as described in Methods.

Line	**Biocatalysts**	**Activity (U/g)**			
1		***p*-NPB**	**Triacetin**	***R*-Methyl Mandelate**	***S*-Methyl Mandelate**
2	Octyl-TLL-GA	1350 ± 90	48± 2	0.8 ± 0.1	2.4 ± 0.1
3	Octyl-TLL-GA-ZnP	1410 ± 80	50 ± 2	0.7 ± 0.1	3.0 ± 0.1
4	Octyl-TLL-GA-CoP	1130 ± 60	35 ± 2	0.2 ± 0.1	2.0 ± 0.1
5	Octyl-TLL-GA-CuP	1140 ± 40	38 ± 2	0.6 ± 0.1	2.1 ± 0.1
6	Octyl-TLL-GA-NiP	1310 ± 50	48 ± 2	0.6 ± 0.1	2.2 ± 0.1
7	Octyl-TLL-GA-MgP	1400 ± 120	46 ± 2	0.7 ± 0.1	2.4 ± 0.1
8	Octyl-TLL-Amin	1670 ± 50	88 ± 4	0.6 ± 0.1	3.2 ± 0.2
9	Octyl-TLL-Amin-ZnP	1420 ± 210	78.± 4	0.6 ± 0.1	3.1 ± 0.2
10	Octyl-TLL-Amin-CoP	1650 ± 90	53 ± 3	0.1 ± 0.1	2.2 ± 0.1
11	Octyl-TLL-Amin-CuP	1620 ± 40	79 ± 4	0.9 ± 0.4	3.0 ± 0.2
12	Octyl-TLL-Amin-NiP	1860 ± 110	63 ± 3	0.6 ± 0.1	2.8 ± 0.1
13	Octyl-TLL-Amin-MgP	1980 ± 90	79 ± 4	0.8 ± 0.1	2.9 ± 0.1
14	Octyl-TLL-TNBS	1760 ± 80	60 ± 3	0.9 ± 0.1	3.1 ± 0.2
15	Octyl-TLL-TNBS-ZnP	1840 ± 100	47 ± 2	0.9 ± 0.1	2.7 ± 0.1
16	Octyl-TLL-TNBS-CoP	1590 ± 110	46 ± 2	0.4 ± 0.1	2.4 ± 0.1
17	Octyl-TLL-TNBS-CuP	1500 ± 80	45 ± 2	1.0 ± 0. 1	3.0 ± 0.2
18	Octyl-TLL-TNBS-NiP	1590 ± 130	44 ± 2	1.4 ± 0.1	3.1 ± 0.2
19	Octyl-TLL-TNBS-MgP	1540 ± 60	46 ± 2	1.1 ± 0.1	3.0 ± 0.2

* ZnP, CuP, CoP, NiP, MgP ZZnP, CuP, CoP, NiP, MgP correspond to the metallic salt/sodium phosphate.

Regarding the effect on the enzyme stability, the stabilizing effect of glutaraldehyde made increasing the temperature to 75 °C to visualize the inactivation of the different biocatalysts in a reasonable timeframe necessary (Figure 7.3a). Zn^{2+} treatment maintained its great stabilization effect, Co^{2+} and Ni^{2+} produced a slight stabilization, Mg^{2+} mineralization

almost had no effect, while copper produced a slight destabilization. In this regard, the situation was not very different to the effects detected using the unmodified biocatalyst. Considering the previous stabilization achieved using glutaraldehyde, the additive effect of the zinc treatment produced a much-stabilized biocatalyst (Figure 7.3a). The glutaraldehyde+Zn^{2+}-treated biocatalyst maintained levels of residual activity similar to the only mineralized biocatalysts after 4 h, but at 7 °C higher temperature (Figures 7.1 and 7.2). It seems that the positive effect from glutaraldehyde on enzyme stability were additive to those of zinc mineralization.

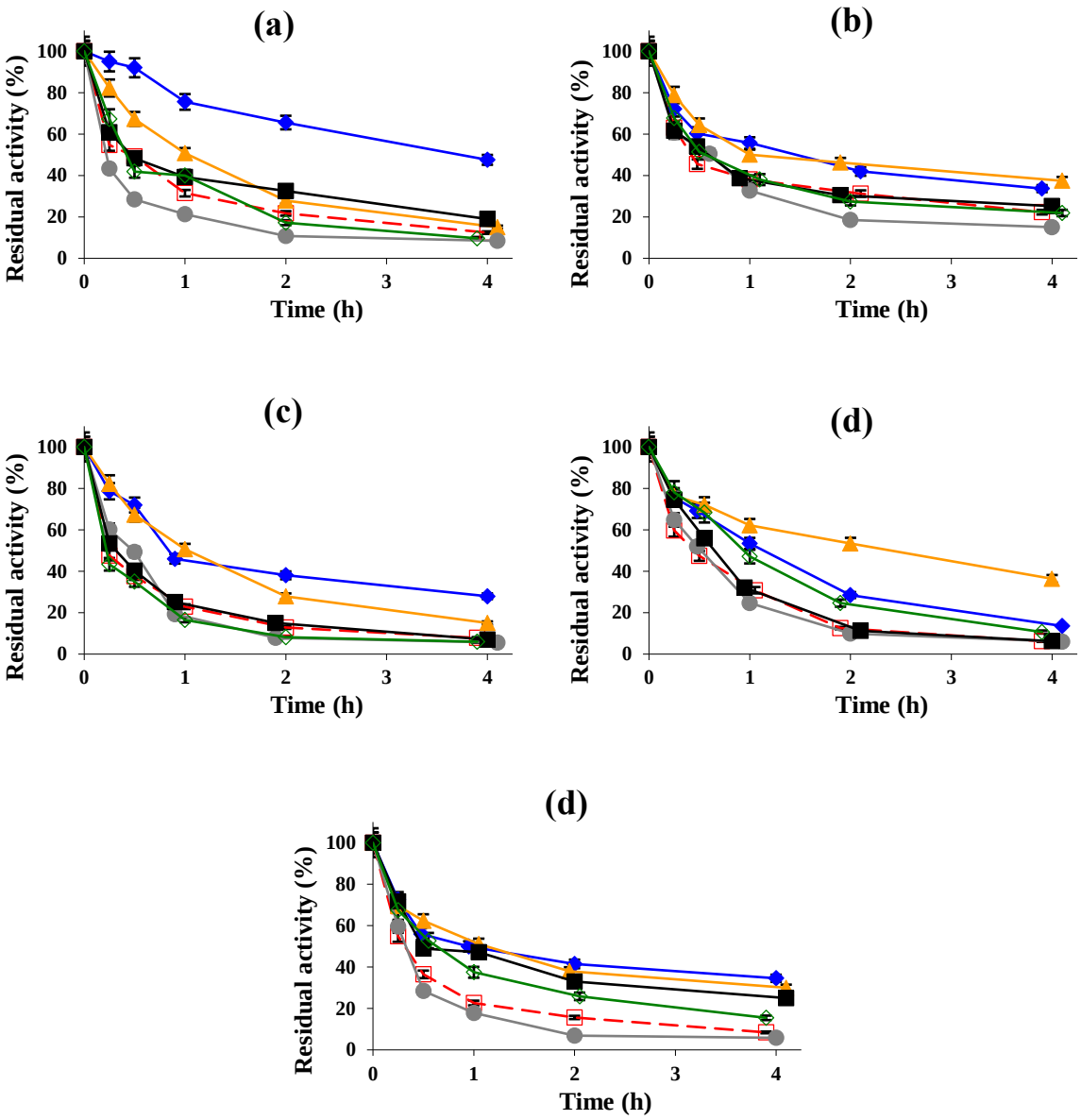

Figure 7.3. Inactivation courses of different octyl-TLL biocatalysts modified with (a) 1% glutaraldehyde, (b) amination using 2 M ethylenediamine, (c) 1 mM picrylsulfonic acid, (d) 10% polyethyleneimine, and (e) 10% dextran sulfate. The biocatalysts were inactivated at 68

°C (b) and 75 °C (a, c, d, e), in presence of 10 mM Tris-HCl buffer at pH 7.0. Other specifications are described in Methods. Unmodified octyl-TLL (empty squares and dashed and red line); octyl-TLL modified with 1% glutaraldehyde (solid rhombus and solid blue line); amination using 2 M ethylenediamine (solid triangles and solid orange line); 1 mM picrylsulfonic acid (solid circles and solid grey line); 10% polyethylenimine (solid squares and solid black line); 10% dextran sulfate (empty rhombus and solid green line).

The mineralization of the aminated biocatalyst gave similar colors to those of the unmodified biocatalysts (Figure A7.2). It produced also significant changes on enzyme specificity (Table 7.3, Line 8-13). Activity using *p*-NPB assay was not too altered, producing a slight decrease on the enzyme activity in most cases (by a maximum of 15% using Cu^{2+}) except a very slight increase using Ni^{2+} (by less than 5%). Using triacetin as substrate, again all mineralized biocatalysts decreased their activity (being the most deleterious modifications those performed using with cobalt, that decreased the activity from 88 U/g to 5.3 U/g). Using *R*-methyl mandelate the enzyme activity remained almost unaltered when mineralized with Ni^{2+} and Zn^{2+}, while drastically decreasing using Co^{2+} (from 0.6 to 0.1 U/g) and increasing when the modification is performed using Cu^{2+} (by 50%) or Mg^{2+} (by 35%). Using the *S*-isomer, all mineralized biocatalysts decreased their activity slightly, except using Co^{2+} that produced a decrease in the enzyme activity from 3.2 to 2.2. These activity changes were translated in significant changes on enzyme specificity and enantiospecificity (Table 7.3, Line 8-13). Regarding the effects on enzyme stability (inactivation was at 68 °C, as amination was negative for enzyme stability), Figure 7.3b shows that Zn^{2+} and Co^{2+} mineralization produced a small stabilization of the biocatalyst, while treatment with Mg^{2+} and Ni^{2+} left the stability unaltered and Cu^{2+} produced a small destabilization. The effects were much smaller than those found using the unmodified immobilized enzyme (Figure 7.2).

The mineralization of the enzyme modified with TNBS give different color to the initially exhibited by the biocatalysts (Figure A7.2), as these preparations presented a yellow-orange color. The treatment produced a slight decrease in *p*-NPB activity (minimum value was observed for the Cu^{2+} modification, decreasing the activity by 17%) (Table 7.3, Line 14-19). The activity versus triacetin was increased in all cases, being the maximum value that observed using Cu^{2+} mineralization (by almost 1.9) and the minimum one that obtained using Zn^{2+} (by around 1.5). The activities versus both isomers of methyl mandelate were fairly maintained (Table 7.3, Line 14-19). Regarding the stability (Figure 7.3c), it was increased using Zn^{2+} and Co^{2+}, the other mineralizations produced marginal effects.

The biocatalysts coated with PEI presented more intense colors than the unmodified biocatalyst, suggesting that PEI can capture some metal salts (Figure A7.2). These modified biocatalysts slightly increased the activity versus *p*-NPB when using Co^{2+}, Cu^{2+} or Zn^{2+} (this last gave the highest value, increasing the activity by more than 15%) while Ni^{2+} and Mg^{2+} treatment slightly decreased the activity (by around 10%) (Table 7.4, Line 2-7). Using triacetin, again all biocatalysts increased their activity, and again Cu^{2+} mineralization gave the highest value (increasing the activity by less than 55%). Using methyl mandelate isomers, the results were quite different, using the *R*-isomer, while Zn^{2+} and Co^{2+} almost did not affect enzyme activity, Mg^{2+}, Cu^{2+} and mainly Ni^{2+} treatments decreased the activity (to less than 50% in the last case), while using the *S*-isomer, all biocatalysts increased their activity, being the highest increase of the immobilized enzyme activity those observed using Ni^{2+} and Mg^{2+} mineralization (almost by 50%). Again, the changes in enzyme activity and specificity were quite relevant (Table 7.4, Line 2-7). Figure 7.3d shows the inactivation courses of these biocatalysts. In this instance, the highest stabilization was found using Co^{2+} treatment, being the Mg^{2+} and Zn^{2+} treatment slightly positive, while Cu^{2+} and Ni^{2+} had no clear effects (Figure 7.3d). These results were quite different to those obtained using the unmodified immobilized enzyme (Figure 7.2).

Last, the enzyme coated with DS was also mineralized, with a color intensity similar to that of the unmodified biocatalyst (Figure A7.2). The effects on *p*-NPB activity (Table 7.4, Line 8-13) were negligible. We can remark the decrease observed using Cu^{2+} (by less than 10%). Using triacetin, a general increase in the activity was detected, with maximum values using Mg^{2+} and Cu^{2+} (increased reaching 1.5-fold). The activities using the methyl mandelate were almost unaffected (a 15% increase for the *R*-isomer using Zn^{2+} and less than a 10% increase for the *S*-isomer using Cu^{2+}) (Table 7.4, Line 8-13). Figure 7.3e shows the inactivation courses, most mineralization produced similar stabilization (not very high) and being the lowest stabilization that found when using Mg^{2+}, except when employing Cu^{2+}, which slightly reduced the enzyme stability.

That way, the results show that using octyl-TLL, the combination of chemical modification and mineralization may greatly alter the enzyme features (activity, specificity and stability). All biocatalysts maintained over 90% of their initial activity after incubation at pH 7 and 37 °C for one month (results not shown), that way, even the least stable ones could be utilized for many applications, thanks to the initial high stability of the enzyme immobilized on octyl-agarose. The color remained attached to the support.

Table 7.4. Specific activity of different immobilized and physically modified biocatalysts after solid-phase mineralization. The hydrolytic activity was measured using 1 mM *p*-NPB (pH 7 and 25 °C), 50 mM triacetin (pH 7 and 25 °C), and 50 mM *R*- or *S*-methyl mandelate (pH 7 and 25 °C). Experiments were conducted as described in Methods.

Line	**Biocatalysts**	**Activity (U/g)**			
1		***p*-NPB**	**Triacetin**	***R*-Methyl Mandelate**	***S*-Methyl Mandelate**
2	Octyl-TLL-PEI	1360 ± 60	90 ± 4	9.8 ± 0.5	7.7 ± 0.4
3	Octyl-TLL-PEI-ZnP	1480 ± 20	106 ± 5	8.2 ± 0.4	8.2 ± 0.5
4	Octyl-TLL-PEI-CoP	1440 ± 30	108± 6	7.7 ± 0.4	8.1 ± 0.3
5	Octyl-TLL-PEI-CuP	1350 ± 80	120 ± 6	8.7 ± 0.4	8.0 ± 0.2
6	Octyl-TLL-PEI-NiP	1310 ± 70	94 ± 4	8.5 ± 0.5	7.9 ± 0.3
7	Octyl-TLL-PEI-MgP	1320 ± 50	80 ± 5	8.2 ± 0.4	7.7 ± 0.3
8	Octyl-TLL-DS	980 ± 50	28 ± 1	7.5 ± 0.4	6.5 ± 0.2
9	Octyl-TLL-DS-ZnP	1040± 30	37 ± 2	9.9 ± 0.4	7.3 ± 0.4
10	Octyl-TLL-DS-CoP	1080 ± 50	27± 1	8.1 ± 0.3	6.1 ± 0.2
11	Octyl-TLL-DS-CuP	900 ± 50	30 ± 2	8.9 ± 0.4	7.2 ± 0.4
12	Octyl-TLL-DS-NiP	1040 ± 40	28± 1	8.5 ± 0.5	7.2 ± 0.3
13	Octyl-TLL-DS-MgP	950 ± 20	31 ± 1	8.9 ± 0.4	7.4 ± 0.4

* ZnP, CuP, CoP, NiP, MgP correspond to the metallic salt/sodium phosphate.

7.3.4 Preparation of the immobilized and chemically modified CALB-biocatalysts

Figure A7.3 shows that only 50% of the enzyme was immobilized on the support, confirming that the loading capacity of the support was exceeded and that way, the full support surface will be coated with enzyme molecules.

Next, octyl-CALB was submitted to the different chemical and physical modifications. First, the effect of these modifications on the enzyme activities was analyzed (Table 7.5). The modification with glutaraldehyde produced a slight increase on the *p*-NPB activity (by 15%), while the activity versus triacetin decreased almost by 30%, the activity versus *R*-methyl mandelate decreased by 15% and versus the *S*-isomer by 30%. The amination of the enzyme produced a similar increase for the *p*-NPB assay, while versus triacetin the decrease in activity was over 40%, the activity versus *R*-methyl mandelate increased by 35% and versus the *S*-isomer decreased by almost 30%. The TNBS modification produced again a 15% increase in

the *p*-NPB activity, versus triacetin the activity decreased by almost 35%, and versus the methyl mandelate esters it remained almost unaltered. The coating with PEI produced a more significant increase in the *p*-NPB activity of the biocatalyst (by more than 30%), a small increase on the activity employing triacetin (by less than 10%), a 33% increase using *R*-methyl mandelate and a 15% decrease using the *S*-isomer (Table 7.5). That way, although not with the same intensity than using TLL (Table 7.1), significant changes in enzyme activity, specificity and enantiospecificity could be observed after the chemical modification of octyl-CALB.

Next, the effect of these modifications on the enzyme stability was analyzed (Figure 7.4). Glutaraldehyde and DS modifications permitted to increase enzyme stability, TNBS permitted an initial slowing of the inactivation but later it was faster than that of the unmodified enzyme, while PEI, and mainly EDA modifications were negative for the enzyme stability. These results were very different to those found using TLL (Figure 7.1).

Table 7.5. Specific activity of different CALB biocatalysts with 1 mM *p*-NPB (pH 7 and 25 °C), 50 mM triacetin (pH 7 and 25 °C), and 50 mM *R*- or *S*-methyl mandelate (pH 7 and 25 °C). Experiments were conducted as described in Methods.

Biocatalysts	**Activity (U/g)**			
	***p*-NPB**	**Triacetin**	***R*-Methyl Mandelate**	***S*-Methyl Mandelate**
Octyl-CALB	1150 ± 60	132 ± 6	19.5 ± 0.9	39.0 ± 1.9
Octyl-CALB-GA	1320 ± 20	94 ± 5	16.8 ± 0.9	28.0 ± 1.4
Octyl-CALB-Amin	1310 ± 40	77 ± 3	26.5 ± 1.3	36.3 ± 1.9
Octyl-CALB-TNBS	1340 ± 40	89 ± 7	18.3 ± 0.8	41.1 ± 2.0
Octyl-CALB-PEI	1520± 90	143 ± 9	26.1 ± 1.5	33.3 ± 1.7
Octyl-CALB-DS	1290± 80	121 ± 4	20.0 ± 0.8	43.2 ± 2.2

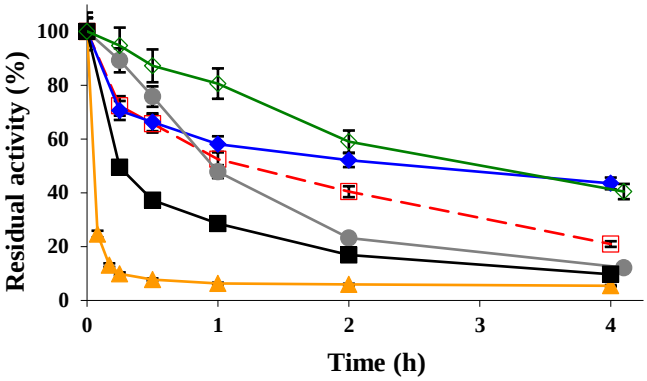

Figure 7.4. Inactivation courses of different physically and chemically modified octyl-CALB biocatalysts. The biocatalysts were inactivated at 75 °C, in presence of 10 mM Tris-HCl buffer at pH 7.0. Other specifications are described in Methods. Unmodified octyl-TLL (empty squares and dashed and red line); octyl-TLL modified with 1% glutaraldehyde (solid rhombus and solid blue line); amination using 2 M ethylenediamine (solid triangles and solid orange line); 1 mM picrylsulfonic acid (solid circles and solid grey line); 10% polyethylenimine (solid squares and solid black line); 10% dextran sulfate (empty rhombus and solid green line).

7.3.5 Mineralization of octyl-CALB

The effect of the enzyme mineralization with different metal phosphates on octyl-CALB activity versus different substrates may be found in Table 7.6. Zn^{2+} treatment maintained the activity versus *p*-NPB, increasing the activity versus triacetin (by around 45%) and both isomers of methyl mandelate (by around 30%). That meant that although enantiospecificity was almost unaltered, the enzyme activity and substrate specificity were quite altered. Regarding the effects on enzyme stability, Figure 7.5 shows that Mg^{2+} and more clearly Ni^{2+} metallization produced some enzyme stabilization, while Zn^{2+} and Cu^{2+} have a small negative effect, that was clearer using Co^{2+}. These results are quite different to those found TLL (Figure 7.2).

That way, even with lower intensity than in the case of TLL, the chemical modification and the mineralization of octyl-CALB produced diverse effects on enzyme specificity, activity, and stability. Next, we have analyzed if the chemical modification can somehow alter the effects of the mineralization on enzyme features, such as occurred using octyl-TLL.

Table 7.6. Specific activity of different CALB biocatalysts with 1 mM *p*-NPB (pH 7 and 25 °C), 50 mM triacetin (pH 7 and 25 °C), and 50 mM *R*- or *S*-methyl mandelate (pH 7 and 25 °C). Experiments were conducted as described in Methods.

Biocatalysts	**Activity (U/g)**			
	***p*-NPB**	**Triacetin**	***R*-Methyl Mandelate**	***S*-Methyl Mandelate**
Octyl-CALB	1150 ± 60	132 ± 6	19.5 ± 0.9	39.0 ± 1.9
Octyl-CALB-ZnP	1170 ± 40	194 ± 7	25.6 ± 1.3	49.5 ± 2.9
Octyl-CALB-CoP	970 ± 50	220 ± 13	27.7 ± 1.4	50.2 ± 2.2
Octyl-CALB-CuP	1000 ± 70	270 ± 14	26.8 ± 1.3	50.1 ± 2.9
Octyl-CALB-NiP	1220 ± 80	222 ± 13	20.4 ± 1.0	43.5 ± 2.1
Octyl-CALB-MgP	1140 ± 50	82 ± 5	17.5 ± 0.9	29.5 ± 1.6

* ZnP, CuP, CoP, NiP, MgP correspond to the metallic salt/sodium phosphate.

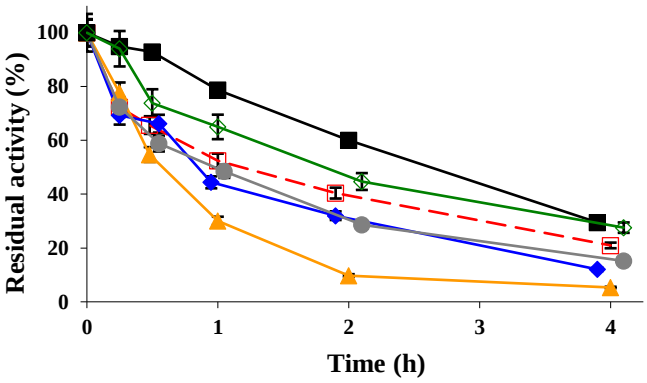

Figure 7.5. Inactivation courses of different octyl-CALB biocatalysts in 10 mM Tris-HCl buffer at pH 7.0 and 75 °C. Other specifications are described in Methods. Unmodified octyl-TLL (empty squares and dashed and red line); octyl-TLL modified with 1% glutaraldehyde (solid rhombus and solid blue line); amination using 2 M ethylenediamine (solid triangles and solid orange line); 1 mM picrylsulfonic acid (solid circles and solid grey line); 10% polyethylenimine (solid squares and solid black line); 10% dextran sulfate (empty rhombus and solid green line).

7.3.6 Effect of the mineralization on the immobilized enzyme previously modified

The mineralization of all preparations gave similar colored biocatalysts (Figure A7.4), being remarkable that the octyl-CALB-PEI presented a more intense color, suggesting a higher metal retention caused to the PEI. The exception is the case of TNBS, as the initial yellow-orange color alters the observed colors after mineralization.

Starting with the enzyme modified with glutaraldehyde, the effect on the *p*-NPB activity is not very significant (Table 7.7, Line 2-7), using Co^{2+} the activity was maintained, using Ni^{2+} a small decrease could be detected (under 5%), being slightly higher the enzyme activity decrease using the other metals (9-12%). The activity versus triacetin slightly decreased for all treatments, being the most significant one the decrease of activity using Ni^{2+} (around 17%). Using *R*-methyl mandelate, the decrease in activity was more significant in some instances, the activity became around 55% for the Zn^{2+} treated biocatalyst and increased more than 10% using Ni^{2+} metalized biocatalyst, the other biocatalysts almost did not alter its activity in this assay. Using the *S*-isomer, Zn^{2+} treated biocatalyst remained the least active biocatalyst, with less than 11% decrease in the enzyme activity, Cu^{2+} treatment increased the activity in a similar way, and the other biocatalysts were in between. That way, the glutaraldehyde modification decreased the effects of the mineralization on the immobilized CALB activity.

Analyzing the effects of the mineralization on the octyl-CALB-GA on enzyme stability (Figure 7.6a), it is easy to visualize that the mineralization effects were also decreased. Zn^{2+}, Co^{2+} and Mg^{2+} presented a negligible positive effect on enzyme stability, while Cu^{2+} and Ni^{2+} produced a slight destabilization, clearer than the stabilization effects of the other metals. The results disagreed with those observed using the unmodified biocatalysts, both in intensity and in quality (e.g., Co^{2+} effects).

Going to the aminated biocatalyst, the effects on the *p*-NPB activity of the mineralization was reduced (Table 7.7, Line 8-13), becoming slightly positive for Ni^{2+} and Cu^{2+} treatments and slightly negative for the other metals (never exceeding 10% variations). Using triacetin, the mineralization produced a general increase of activity. The highest one was that obtained using Co^{2+} and Mg^{2+} (by more than 55%) and the lowest one was that observed after treatment using Cu^{2+} (by around 25%). When using *R*-methyl mandelate, the activities slightly decreased, with minimum values using Zn^{2+} and Mg^{2+} (decreasing the activity to 90-89%). Using the *S*-isomer, only Ni^{2+} treatment increased the activity very slightly (by less than 5%), while the other metals produced a relevant decrease in enzyme activity (to a minimum of 75%) (Table 7.7, Line 8-13). These effects were quite different to those found using glutaraldehyde modified preparations or the unmodified biocatalyst. Figure 7.6b shows that all mineralizations

improved the enzyme stability (not in a very significant way). Co^{2+}, Zn^{2+} and Cu^{2+} treatments showed the best stabilization effects.

Table 7.7. Specific activity of different immobilized and chemically modified CALB biocatalysts after solid-phase mineralization. The hydrolytic activity was measured using 1 mM *p*-NPB (pH 7 and 25 °C), 50 mM triacetin (pH 7 and 25 °C), and 50 mM *R*- or *S*-methyl mandelate (pH 7 and 25 °C). Experiments were conducted as described in Methods.

Line	**Biocatalysts**	**Activity (U/g)**			
1		***p*-NPB**	**Triacetin**	***R*-Methyl Mandelate**	***S*-Methyl Mandelate**
2	Octyl-CALB-GA	1320 ± 20	94 ± 5	16.8 ± 0.9	28.0 ± 1.4
3	Octyl-CALB-GA-ZnP*	1190 ± 20	92 ± 5	9.7 ± 0.5	25.0 ± 1.9
4	Octyl-CALB-GA-CoP	1320 ± 40	85 ± 4	15.5 ± 0.8	26.6 ± 1.3
5	Octyl-CALB-GA-CuP	1190 ± 30	82 ± 4	14.1 ± 0.7	31.4 ± 1.4
6	Octyl-CALB-GA-NiP	1260 ± 40	78 ± 2	18. 9 ± 0.8	27.8 ± 1.2
7	Octyl-CALB-GA-MgP	1260 ± 60	121 ± 6	23.8 ± 1.0	29.2 ± 1.6
8	Octyl-CALB-Amin	1310 ± 40	77 ± 3	26.5 ± 1.3	36.3 ± 1.9
9	Octyl-CALB-Amin-ZnP	1270 ± 20	112 ± 7	23.6 ± 1.4	27.9 ± 1.3
10	Octyl-CALB-Amin-CoP	1220 ± 70	122± 6	25.3 ± 1.1	27.0 ± 1.0
11	Octyl-CALB-Amin-CuP	1340 ± 60	97 ± 6	24.3 ± 0.9	27.6 ± 1.2
12	Octyl-CALB-Amin-NiP	1370 ± 100	116 ± 6	24.7 ± 1.4	37.0 ± 1.9
13	Octyl-CALB-Amin-MgP	1260 ± 60	121 ± 6	23.8 ± 1.0	29.2 ± 1.6
14	Octyl-CALB-TNBS	1340 ± 40	89 ± 7	18.3 ± 0.8	41.1 ± 2.0
15	Octyl-CALB-TNBS-ZnP*	1270 ± 70	139± 7	16.7 ± 0.8	39.1 ± 2.0
16	Octyl-CALB-TNBS-CoP	1260 ± 80	140 ± 8	17.4 ± 0.7	37.5 ± 1.9
17	Octyl-CALB-TNBS-CuP	1130 ± 60	169 ± 8	19.3 ± 0.9	43.3 ± 2.1
18	Octyl-CALB-TNBS-NiP	1210 ± 120	153 ± 7	20.0 ± 0.8	38.2 ± 1.9
19	Octyl-CALB-TNBS-MgP	1170 ± 30	145 ± 9	18.6 ± 1.2	39.8 ± 2.0

* ZnP, CuP, CoP, NiP, MgP correspond to the metallic salt/sodium phosphate.

The mineralization of the TNBS modified CALB biocatalyst produced a slight decrease in *p*-NPB activity (by 13% in the highest case, using Mg^{2+}) (Table 7.7, Line 14-19) but relevantly increased the activity versus triacetin in all cases, being the treatment with the highest

increase of immobilized enzyme activity that the one obtained using Cu^{2+} (1.9-fold) and the lowest, that using Zn^{2+} and Co^{2+} (more than 1.5-fold). Using both isomers of methyl mandelate, the changes never exceeded 10%. The effects on enzyme stability may be visualized on the inactivation courses represented in Figure 7.6c, only Ni^{2+} treated biocatalyst slightly decreased its stability, all the other biocatalysts had not significant differences.

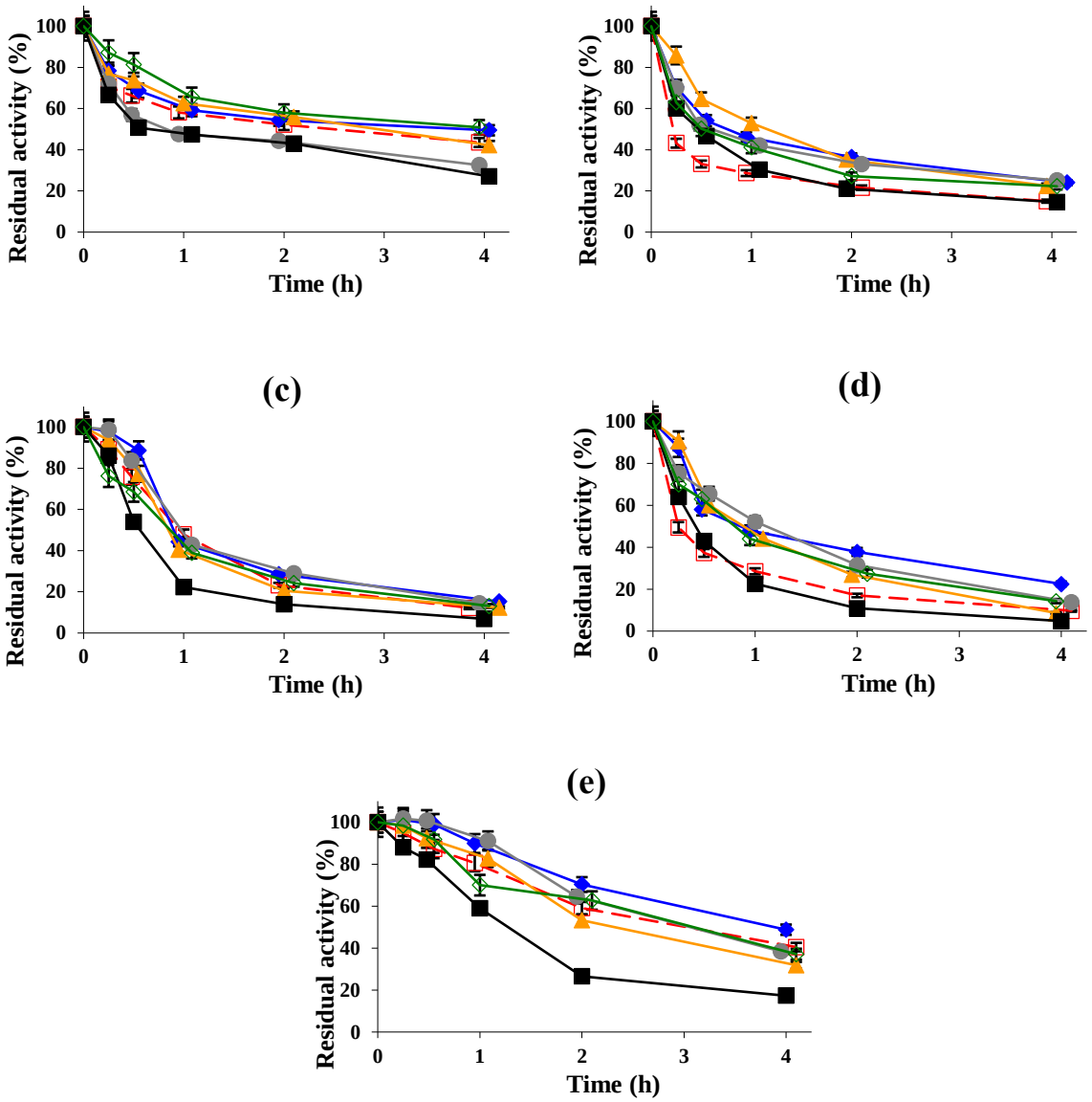

Figure 7.6. Inactivation courses of different octyl-CALB biocatalysts modified with (a) 1% glutaraldehyde, (b) amination using 2 M ethylenediamine, (c) 1 mM picrylsulfonic acid, (d) 10% polyethylenimine, and (e) 10% dextran sulfate. The biocatalysts were inactivated at 68 °C (b) and 75 °C (a, c, d, e), in presence of 10 mM Tris-HCl buffer at pH 7.0. Other specifications are described in Methods. Unmodified octyl-TLL (empty squares and dashed and red line);

octyl-TLL modified with 1% glutaraldehyde (solid rhombus and solid blue line); amination using 2 M ethylenediamine (solid triangles and solid orange line); 1 mM picrylsulfonic acid (solid circles and solid grey line); 10% polyethylenimine (solid squares and solid black line); 10% dextran sulfate (empty rhombus and solid green line).

Table 7.8. Specific activity of different immobilized and physically modified CALB biocatalysts after solid-phase mineralization. The hydrolytic activity was measured using 1 mM *p*-NPB (pH 7 and 25 °C), 50 mM triacetin (pH 7 and 25 °C), and 50 mM *R*- or *S*-methyl mandelate (pH 7 and 25 °C). Experiments were conducted as described in Methods.

Line	**Biocatalysts**	**Activity (U/g)**			
1		***p*-NPB**	**Triacetin**	***R*-Methyl Mandelate**	***S*-Methyl Mandelate**
2	Octyl-CALB-PEI	1520 ± 90	143 ± 9	26.1 ± 1.5	33.3 ± 1.7
3	Octyl-CALB-PEI-ZnP	1770 ± 30	181 ± 7	26.9 ± 1.4	38.2 ± 2.0
4	Octyl-CALB-PEI-CoP	1640 ± 30	201 ± 11	26.3 ± 1.4	40.8 ± 2.1
5	Octyl-CALB-PEI-CuP	1660 ± 50	219 ± 14	17.2 ± 0.9	45.9 ± 2.9
6	Octyl-CALB-PEI-NiP	1490 ± 90	200± 11	12.5 ± 0.7	47.8 ± 2.3
7	Octyl-CALB-PEI-MgP	1470 ± 90	207 ± 13	19.1 ± 1.0	48.5 ± 2.6
8	Octyl-CALB-DS	1290 ± 80	121 ± 4	20.0 ± 0.8	43.2 ± 2.2
9	Octyl-CALB-DS-ZnP	1250 ± 50	132 ± 8	23.6 ± 1.1	43.2 ± 1.9
10	Octyl-CALB-DS-CoP	1250 ± 60	144 ± 8	21.4 ± 1.0	44.8 ± 2.6
11	Octyl-CALB-DS-CuP	1190 ± 100	182 ± 10	20.9 ± 0.8	46.6 ± 2.5
12	Octyl-CALB-DS-NiP	1230 ± 30	159 ± 9	19.3 ± 0.6	41.0 ± 1.9
13	Octyl-CALB-DS-MgP	1310 ± 30	179 ± 10	21.5 ± 0.9	44.3 ± 1.4

* ZnP, CuP, CoP, NiP, MgP correspond to the metallic salt/sodium phosphate.

Using PEI coated biocatalyst, the enzyme activity versus *p*-NPB increased using Zn^{2+} (by more than 15%) (Table 7.8, Line 2-7). As this was an additive to the increase of immobilized enzyme activity obtained in the PEI coating, this converted this biocatalyst in the most active versus this substrate. Also, Co^{2+} and Cu^{2+} mineralization increased the enzyme activity (by 7-8%). However, the modifications with Ni^{2+} and Mg^{2+} had no effects. Using triacetin, all mineralizations improved the enzyme activity, by around 50% except using Zn^{2+} (this gave an activity of 125%). The activity versus *R*-methyl mandelate decreased using Mg^{2+} (to 73%),

Cu^{2+} (to 66%) and Ni^{2+} (to 48%), while the other two metals had no relevant effects. In the activity detected using the *S*-isomer, all biocatalysts increased their activities, being the most active those treated with Cu^{2+} and Mg^{2+} (increasing the activity by 45%) (Table 7.8, Line 2-7). This promoted large changes in enzyme specificity, activity and enantiospecificity, different to those detected mineralizing the unmodified biocatalyst (Table 7.5). Regarding the effects on enzyme stability, Figure 7.6d shows that the mineralization with Ni^{2+} presented a slightly negative effect, while all the other slightly improved enzyme stability (increasing the half live by 2 to 4-fold).

Using DS coated biocatalyst; the enzyme *p*-NPB activity did not change in a relevant way, except for the Cu^{2+} treated biocatalyst, that lost almost 9% of its activity (Table 7.8, Line 8-13). The activity using triacetin increased in all cases, with the highest increase of immobilized enzyme activity around 50% using Cu^{2+} and Mg^{2+}. The activity versus *R*-methyl mandelate was maintained or even increased (by 18% using Zn^{2+}), in a similar trend to the unmodified biocatalyst and differently to the other modified biocatalyst. Using the *S*-isomer, its activity was maintained or slightly increased (by a maximum of only 6%). This was different to the unmodified biocatalyst, where the activity increased in a more significant way and for more metalizations (see Table 7.6). Enzyme stability was significantly decreased using Ni^{2+} treatment, while the treatments with the other metals had scarce effects (Figure 7.6e).

7.4 Conclusions

This paper shows, using two enzymes, how the previous physicochemical modification of immobilized lipases strongly affects the effects of mineralization on their catalytic properties. TLL was found to be more tunable than CALB, perhaps because of the smaller lid that CALB exhibits [114], in any case this enzyme usually is less tunable than other lipases (e.g., by the immobilization conditions) [115].

The effect of the same metallization may be positive for the biocatalyst activity versus one substrate and negative for the activity versus other substrate and this effect depends on the enzyme and on their previous physicochemical modification. Similarly, the stability of the enzymes is altered by both physicochemical modification and mineralization, and these effects are not directly translated from one enzyme or biocatalysts to another. Mineralization of chemically or physically modified immobilized enzymes is a potent tool to improve enzyme features, but the effects cannot be predicted at this stage and must be empirically analyzed.

Investigating the actual causes of these effects would be a very interesting target. However, considering that the metal phosphate form crystals also inside supports without enzyme [32], the analysis may be hard. It could be highly interesting to investigate the different structural changes that the enzyme can adopt due to the different chemical and physical modifications, as this can help to the different activities and stabilities of the biocatalysts [104]. Unfortunately, although the potential of spectroscopic physico-chemical and biophysical techniques to advance in the structural analysis of immobilized enzymes and understand the structure-function relationship of the enzymes is advancing very rapidly [116–125], nowadays they have many limits [116,117]. That way, these techniques are still far from providing a clear and unique vision that can explain the functional features of an immobilized enzyme, even though this should be of great interest to improve the understanding the phenomena that determine the final properties of the immobilized enzyme and this may open new opportunities in the development more controllable and efficient immobilization process.

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APPENDIX

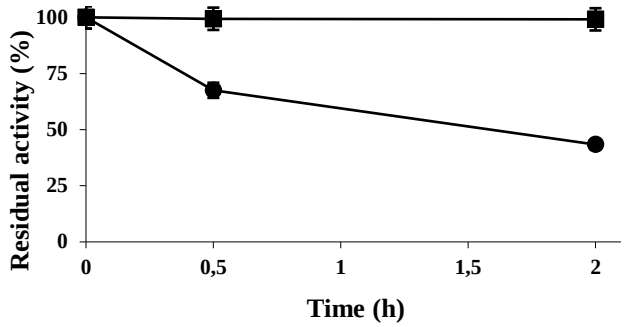

Figure A7.1. Immobilization course of TLL over octyl agarose using enzyme loading of 20 mg/g. The immobilization was performed in 5 mM sodium phosphate at 25 °C and pH 7.0. Squares: reference and circles: supernatant. Other specifications are described in Methods.

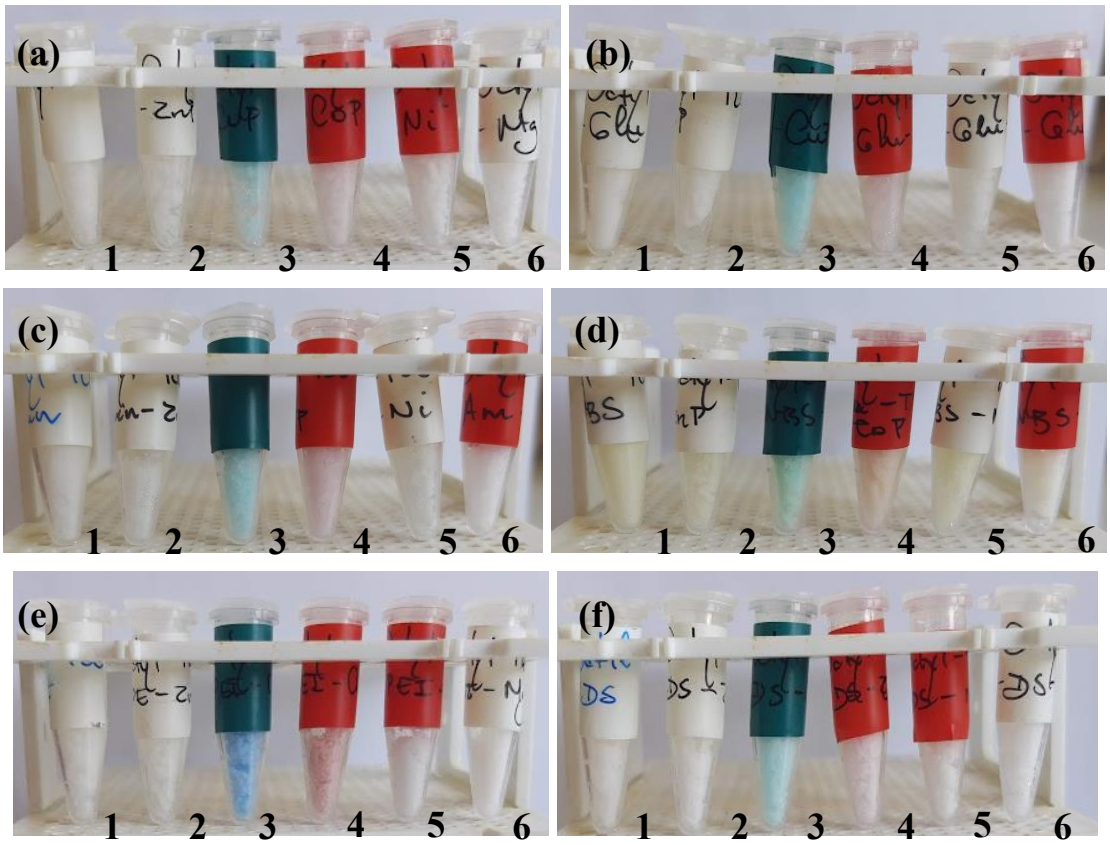

Figure A7.2. (a) Octyl-TLL biocatalysts modified with (b) 1% glutaraldehyde, (c) amination using 2 M ethylenediamine, (d) 1 mM picrylsulfonic acid, (e) 10% polyethylenimine, and (f) 10% dextran sulfate (1) untreated and (2-6) treated with (2) $ZnCl_2$/sodium phosphate, (3) $CuCl_2$/sodium phosphate, (4) $CoCl_2$/sodium phosphate, (5) $NiCl_2$/sodium phosphate, and (6) $MgCl_2$/sodium phosphate. Other specifications are described in Methods.

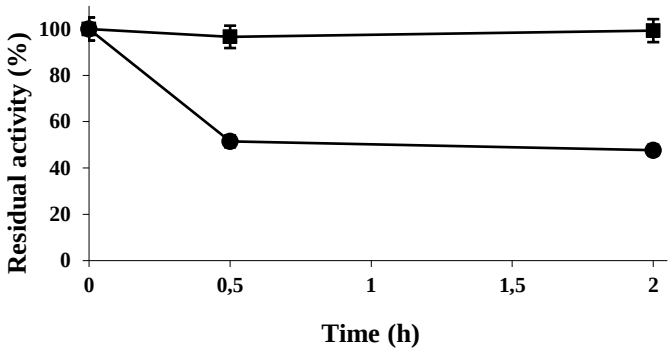

Figure A7.3. Immobilization course of CALB over octyl agarose using enzyme loading of 20 mg/g. The immobilization was performed in 5 mM sodium phosphate at 25 °C and pH 7.0. Squares: reference and circles: supernatant. Other specifications are described in Methods.

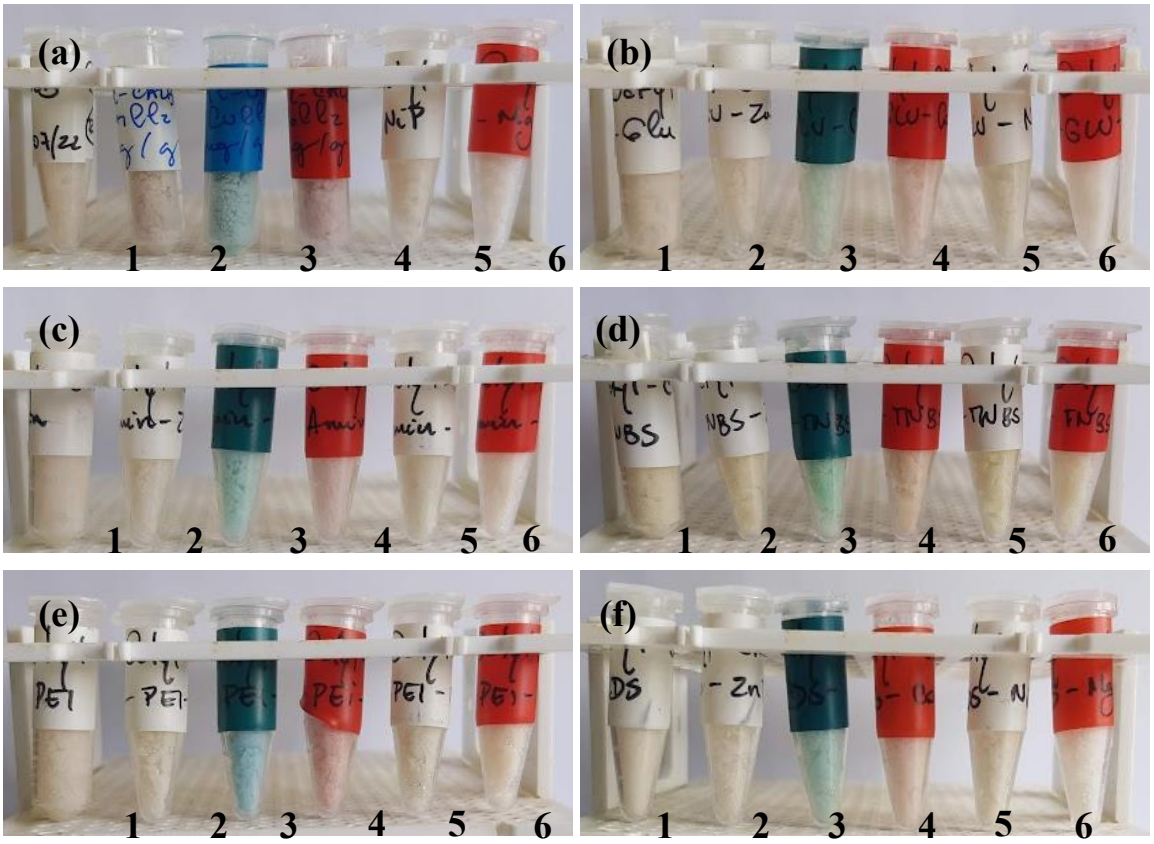

Figure A7.4. (a) Octyl-CALB biocatalysts modified with (b) 1% glutaraldehyde, (c) amination using 2 M ethylenediamine, (d) 1 mM picrylsulfonic acid, (e) 10% polyethylenimine, and (f) 10% dextran sulfate (1) untreated and (2-6) treated with (2) $ZnCl_2$/sodium phosphate, (3) $CuCl_2$/sodium phosphate, (4) $CoCl_2$/sodium phosphate, (5) $NiCl_2$/sodium phosphate, and (6) $MgCl_2$/sodium phosphate. Other specifications are described in Methods.

CHAPTER 8
HETEROFUNCTIONAL METHACRYLATE BEADS BEARING OCTADECYL AND VINYL SULFONE GROUPS: TRICKS TO GET AN INTERFACIALLY ACTIVATED LIPASE FROM *Thermomyces lanuginosus* AND COVALENTLY ATTACHED TO THE SUPPORT

Lipase from *Thermomyces lanuginosus* (TLL) has been immobilized on a methacrylate macroporous resin coated with octadecyl groups (Purolite Lifetech® ECR8806F). This immobilization protocol gave a biocatalyst with a significantly higher stability than that obtained using octyl agarose. To further improve the biocatalyst features, we tried to covalently immobilize the enzyme using this support. For this purpose, the support was activated with divinyl sulfone. The results showed that at least 1/3 of the immobilized enzyme was not covalently immobilized. To solve the problem, we produced an aminated support and then, activated it with divinyl sulfone. This permitted the full covalent immobilization of the previously immobilized TLL. The use of different blocking agents as reaction end point (using ethylenediamine, aspartic acid, glycine, and cysteine) greatly altered the biocatalyst functional features (activity, specificity, or stability). For example, the blocking with ethylenediamine increased the ratio of the activity versus *R*- and *S*-methyl mandelate by a 3-fold factor. The blocking with Cys produced the most stable biocatalyst, maintaining close to 90% of the activity under conditions where the just adsorbed enzyme maintained less than 55%. That way, this strategy to modify the support has permitted to get an enzyme interfacially activated versus the octadecyl layer and later, covalently immobilized by reaction with the vinyl sulfone groups.

8.1 Introduction

Lipases are very interesting biocatalysts, as they lack cofactors, have a low specificity coupled in some instances with a high regio- or enantio- selectivity or specificity, and present a good stability in different reaction media [1–5]. That way, lipases have been utilized in a wide range of processes, from commodities production to food chemistry, from energy to fine chemistry [5–12]. Even using these robust enzymes, they have some deficiencies due to their adaptation to fulfil their physiological function. That way, their very good catalytic properties are mainly addressed for their physiological substrates and under physiological conditions, and they can be inactivated, inhibited, etc. by different causes. Among the different tools to improve enzyme features (metagenomics [13,14], directed evolution [6,15–17], site directed

mutagenesis [18,19], chemical modification [20]), in this paper we will focus on enzyme immobilization. It started as a technique to solve the problems derived from enzyme solubility, that makes enzyme recovery and reuse complex [21]. Having a heterogeneous biocatalyst also facilitates reaction control and enlarges the type of reactors that can be used. Nowadays, with the price of the enzymes going down due to the rapid development of enzyme expression and overproduction technologies, enzyme immobilization must fulfill other requirements [22–24]. In fact, a proper immobilization may improve the enzyme stability by diverse reasons, as recently reviewed [25]. Moreover, immobilization may be coupled also to enzyme purification if the immobilization protocol is designed to reach this goal. Enzyme immobilization may alter the enzyme conformation, places the enzyme in a confined space and in a special environment, and all these can affect enzyme activity, selectivity, specificity, and inhibitions. That way, enzyme immobilization has become a potent tool to design a suitable industrial biocatalyst. However, this is an area that still requires an intense investigation, as the results depend on many factors and some of them may be unknown nowadays [26].

The case of lipases is special. They are interfacial enzymes (enzymes able to act in the water/oil drop interface) [27]. This is because of the peculiar conformation of their active center, that is usually isolated from the medium by a polypeptide chain called lid, which has some mobility and can move exposing the active center (open conformation) [28–33]. This open conformation expos a large hydrophobic pocket and tends to become adsorbed to any hydrophobic surface, such as the natural substrate, a drop of oil. Moreover, lipases have a similar tendency to become adsorbed on any other hydrophobic surface: a hydrophobic protein [34,35], the open form of other lipase [36] or a hydrophobic support. This has promoted that the immobilization of lipases on hydrophobic supports has become a very popular strategy, as it permits to immobilize, purify, stabilize and hyperactivate lipases in one step, ensuring the existence of just one lipase form, the open and monomeric form [37,38]. This immobilization strategy, although giving quite strongly adsorbed lipases, is reversible, and the enzyme may be released to the medium in certain circumstances: high temperature, presence of solvents [39], presence of substrate or products with detergent-like properties (such as partial glycerides or free fatty acids) [40,41], etc. This has been solved using heterofunctional supports, having a layer of acyl groups to get the lipase interfacial activation and a layer of groups able to give other physical interactions (e.g., ionic exchange [42]) or a covalent bond, that will make the immobilization irreversible. The covalent bonds have been tried using glyoxyl, glutaraldehyde or vinyl sulfone groups [39,43–51]. These last, due to their longer spacer arm and reactivity with very different nucleophilic groups in the enzyme surface, gave the best results [43,52].

Furthermore, the blocking step required using this immobilization strategy gave the opportunity to generate special enzyme-support interactions, which fully altered the enzyme features [43,53,54] and even the inactivation pathway [55]. These strategies have been mostly employed when agarose was the support, and the acyl group was octyl.

In this context, it has been recently shown that the immobilization of the lipase from *Thermomyces lanuginosus* (TLL) on methacrylate beads bearing octadecyl groups allowed obtaining a biocatalyst with very good features in biodiesel production, becoming in the same order of magnitude to the reaction rate using alkaline catalyst [56–58]. In this new research effort, we intend to prepare a TLL biocatalyst using this support, activating the support surface with divinyl sulfone to achieve covalently immobilized biocatalysts after the first immobilization via interfacial activation, to further improve the enzyme features. The difficulties are two: the activation of this support with divinyl sulfone groups that should be exposed to the medium can be complex, and even in the best case, the long octadecyl groups can generate great steric hindrances for the reaction with the immobilized enzymes. One alternative is to transform the support in an aminated one, using this amino groups in the modification with divinyl sulfone, this can permit to have more reactive groups and with a longer spacer arm. Also, even hindered by the layer of octadecyl group, this layer of aminated groups can give a new ion exchange capacity to the support [59,60], (it will be a trifunctional one bearing octadecyl, amino and vinyl sulfone (VS) groups), that can approximate the enzyme to the support surface.

8.2 Materials and Methods

8.2.1 Materials

Liquid TLL formulation with 20.8 mg protein/mL (kindly donated by Novozymes Spain (Madrid, Spain)) was utilized in this paper. Bradford´s reagent (utilized to calculate the protein concentration [61]), *p*-nitrophenyl-butyrate (*p*-NPB), triacetin, *R*- and *S*-methyl mandelate, acetonitrile for HPLC (gradient grade, purity ≥ 99.9%), sodium periodate, sodium borohydride, ethylenediamine (EDA), and the amino acids glycine (Gly), aspartic acid (Asp), and cysteine (Cys) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Divinyl-sulfone (DVS) was purchased from Thermo Fisher Scientific Spain (Madrid, Spain). Purolite Lifetech® ECR8806F (methacrylate macroporous resin containing octadecyl - C18 - groups) (Purolite® C18) was kindly donated from Purolite® Ltd. (Wales, UK). All other reagents were of analytical grade.

Elemental analyses were performed by CAI de Microanálisis Elemental, Universidad Complutense, using a Leco 932 CHNS combustion microanalyzer.

8.2.2 Methods

All experiments were performed at least by triplicate, and the values are presented as mean values and standard deviation.

8.2.2.1 Wetting of Purolite® C18 beads

Prior to use, 1 g of Purolite® C18 beads were suspended in 5 mL of methanol and kept under gentle agitation for 1 h to remove the air inside the particles [56]. Subsequently, 5 mL of distilled water was added, maintaining the stirring for 15 min. Afterwards, the suspensions were vacuum filtered, washed 10 times with 20 volumes of distilled water, and stored at 4-6 °C.

8.2.2.2 Preparation of octadecyl-vinyl sulfone Purolite® beads

The octadecyl-vinyl sulfone (VS) Purolite support was prepared under two conditions following the methodology described by Albuquerque et al. [43].

In the first condition, 10 g of Purolite® C18 beads was added to 200 mL of 350 mM divinyl-sulfone prepared in 333 mM sodium carbonate at pH 11.5. The suspension was incubated at room temperature under gentle stirring for 2 h. After, the vinyl-sulfone-octadecyl support was vacuum filtered, washed 10 times with 20 volumes of distilled water, and stored at 4-6 °C.

In the second condition, an amount of 200 μmol of sodium periodate per g of support was added to a suspension of Purolite® C18 beads (0.1 g/mL) prepared in distilled water to oxidize the support producing aldehyde groups. The oxidation process was monitored by spectroscopy at 450 nm (isosbestic point), adding 100 microliters of the oxidation suspension to a mixture of 1 mL of sodium bicarbonate saturated solution and 1 mL of 1 M potassium iodide. Oxidation took place under gentle agitation for 3 h at 25 °C. Then, the suspension was vacuum filtered and washed 10 times with 20 volumes of distilled water. After recovery, the support was resuspended in 2 M EDA at pH 10.0 (1:10 ratio, w/v). The suspension was incubated with gentle agitation for 48 h. Then, solid sodium borohydride was added to reach a concentration of 10 mg/mL. The reduction was carried out under gently stirring for 30 min. Next, the suspension was vacuum filtered and washed 10 times with 20 volumes of distilled water to recover the activated support. After, 10 g of the aminated support was added to 200 mL of 350 mM divinyl-sulfone prepared in 333 mM sodium carbonate at pH 11.5 under gently

stirring for 2 h. Subsequently, the support was vacuum filtered, washed 10 times with 20 volumes of distilled water, and stored at 4-6 °C.

8.2.3 TLL immobilization

8.2.3.1 Immobilization of lipases on wet Purolite® C18 beads

TLL was immobilized by interfacial activation in previously hydrated Purolite® C18 beads using an enzyme loads of 20 mg/g. An excess of enzyme was utilized to ensure that the support surface was fully coated with the enzyme. 10 g of support was added to 100 mL of enzyme solution prepared in 5 mM sodium phosphate at pH 7.0. The immobilization was conducted at room temperature under gentle stirring for 2 h, measuring the activity in supernatant and reference using *p*-NPB assay. Afterwards, the suspensions were vacuum filtered, washed 10 times with 20 volumes of distilled water, and stored at 4-6 °C.

8.2.3.2 Immobilization of lipases on octadecyl-vinyl sulfone Purolite® beads

10 g of support was added to 10 mL of enzyme solution prepared in 5 mM sodium acetate at pH 5.0 (enzyme loads of 20 mg/g, exceeding the maximum load capacity of the support in purpose). The pH 5.0 was used to favor interfacial activation as the main first cause for enzyme immobilization [43]. The immobilization was conducted at room temperature under gentle stirring for 2 h, measuring the activity in supernatant and reference using *p*-NPB assay. The enzyme immobilized on this support almost expressed no activity versus this substrate. Afterwards, the suspensions were vacuum filtered and washed 10 times with 20 volumes of distilled water. After recovery, 1 g of the immobilized enzyme was suspended in 10 mL of 100 mM sodium carbonate at pH 10.0. The suspension was incubated at room temperature for 24 h to favor the formation of covalent bonds between enzyme-support. Afterwards, the biocatalysts were vacuum filtered. After recovery, 1 g of biocatalyst was added to 10 mL of 2 M blocking agent (Gly, Asp, Cys, or EDA) at pH 10.0. The modification of remaining vinyl sulfone was carried out at 25 °C under gentle agitation for 48 h. Subsequently, the support was vacuum filtered, washed 10 times with 20 volumes of distilled water, and stored at 4-6 °C.

8.2.4 SDS-PAGE analysis

SDS-PAGE analyses were performed following the methodology described by Laemmli [62]. Samples were suspended in rupture buffer (0.1 g immobilized enzyme/mL of solution) and boiled for 5 min. After this period, the support was centrifuged at 10,000 rpm for 5 min.

Proteins not covalently immobilized to the support were released into the supernatant by this treatment [63]. 14 μL aliquots of the supernatant were used to perform SDS-PAGE analysis at 100 V. 5 μL of low molecular weight marker proteins (LMW-SDS Marker 14.4-97 kDa) were used as a standard. The gel was stained using Coomassie brilliant blue.

8.2.5 Thermal inactivation of the different TLL preparations

In a standard experiment, the immobilized biocatalyst was suspended in 10 mM Tris-HCl (1:10 ratio, w/v) at pH 7.0 and incubated at 75 °C. Phosphate was avoided due to the detrimental effect of this anion on lipases immobilized via interfacial activation [64]. 50 μL of the inactivation suspensions were collected periodically to determine their residual activities. Residual activities were defined as the current activity divided by the initial in percentage. The experiments were carried out using triacetin as a substrate.

8.2.6 Enzyme activity assays

One unit of activity (U) was defined as the amount of enzyme that hydrolyzes one μmol of substrate per minute under the described conditions.

8.2.6.1 Hydrolysis of p-NPB

50 μL of a soluble enzyme solution or supernatant of enzyme suspension (e.g., in enzyme immobilization suspensions) was added to a mixture of 50 mL of 10 mM *p*-NPB prepared in acetonitrile and 2.5 mL of 25 mM sodium phosphate at pH 7.0. The hydrolysis was conducted using a thermostatization system at 25 °C under magnetic stirring for 1.5 min. The *p*-nitrophenol released into the medium was monitored by spectrophotometry at 348 nm (isosbestic point) to determine the hydrolytic activity ($\varepsilon = 5150\ M^{-1}\ cm^{-1}$) [65].

8.2.6.2 Hydrolysis of triacetin

50 mg of immobilized enzyme were added to 3 mL of 50 mM of triacetin prepared in 50 mM of sodium phosphate at pH 7.0. The reaction was carried out at room temperature under gentle stirring. The quantification of hydrolysis was determined by quantifying the released 1,2 diacetin (under these conditions the produced 1,2 diacetin undergoes acyl migration giving 1,3 diacetin) in the reaction medium [66]. A Waters 486 chromatograph (Waters, Millford, USA) presenting a Kromasil C18 column (15 cm x 0.46 cm) and a UV/VIS detector (set to 230 nm) was employed in the analyses to determine the degree of conversion (two points over 5% and under 25%, to ensure linearity and minimize experimental error) and enzymatic activity. The

mobile phase was composed of 85% (v/v) water and 15% (v/v) acetonitrile with a flow rate of 1 mL/min. The retention times were 4 min for 1,2 and 1,3 diacetins (under these conditions, they eluted at the same retention time) and 18 min for triacetin [67].

8.2.6.3 Hydrolysis of R- or S-methyl mandelate

50 mg of immobilized lipase were added to 3 mL of 50 mM *R*- or *S*-methyl mandelate in 50 mM sodium phosphate at pH 7.0. The reaction was carried out at room temperature under gentle stirring. The quantification of hydrolysis degree was determined by the determination of the released mandelic acid in the reaction medium. A Waters 486 chromatograph (Waters, Millford, USA) presenting a Kromasil C18 column (15 cm x 0.46 cm) and a UV/VIS detector (set to 230 nm) was employed in the analyses to determine the degree of conversion (two points over 5% and under 25% conversion, to ensure linearity and minimize experimental error caused by the initial acid content of the samples) and enzymatic activity [55]. The mobile phase was 10 mM ammonium acetate and acetonitrile (65-35% (v/v)) at pH 2.8 with a flow rate of 1 mL/min. The retention times were 2.5 min for mandelic acid and 4.2 min for the *R*- or *S*-methyl mandelate [54]. Activities ratio was defined as activity versus the *R*-isomer/activity versus the *S*-isomer.

8.3 Results and Discussion

8.3.1 Immobilization of TLL on Purolite C18 and Purolite C18-VS

Figure 8.1 shows the immobilization of TLL on Purolite C18 and Purolite C18-VS. In both cases the kinetics are similar, as around 45% of the offered enzyme activity are immobilized, allowing to infer that around 9 mg of enzyme/g of support are immobilized (TLL is pure, as had one electrophoretic band, data not shown). There are no significant increases in the immobilization yield when prolonging the immobilization to 3 h. This result is due to our using on purpose an excess of enzyme, to ensure that the biocatalysts is fully loaded, the non-immobilized enzyme remains fully active and could be reused in new immobilization cycles.

The enzyme immobilized on Purolite C18-VS was incubated at alkaline pH to have some covalent bonds with the support and blocked with Asp. The SDS-PAGE analysis (Figure 8.2) of this biocatalyst at different amounts showed that a high percentage of TLL molecules remained just physically immobilized, about 30% of the enzyme molecules were released from the support [63]. This was a result even worse than using glyoxyl-octyl agarose to immobilize this enzyme [39], a problem that was solved in that case using the heterofunctional octyl-VS-

agarose [43]. This could be caused by the long octadecyl groups, which can promote steric hindrances for the enzyme-support reaction and makes very difficult to establish a covalent bond [68]. That way, we decided to try other strategy to get a full TLL covalent immobilization.

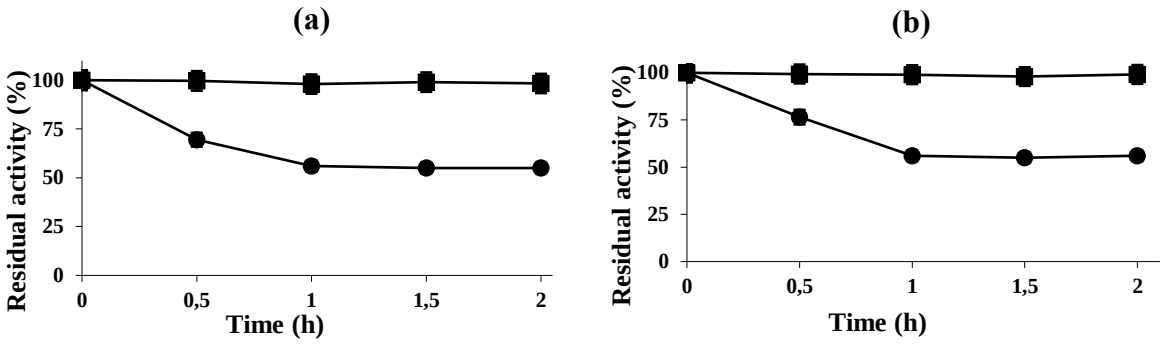

Figure 8.1. Immobilization course of TLL over (a) Purolite C18 beads and (b) Purolite C18-VS beads using an enzyme loading of 20 mg/g. The immobilization was performed in 5 mM sodium acetate at 25 °C and pH 5.0. Squares: reference and circles: supernatant. Other specifications are described in Methods.

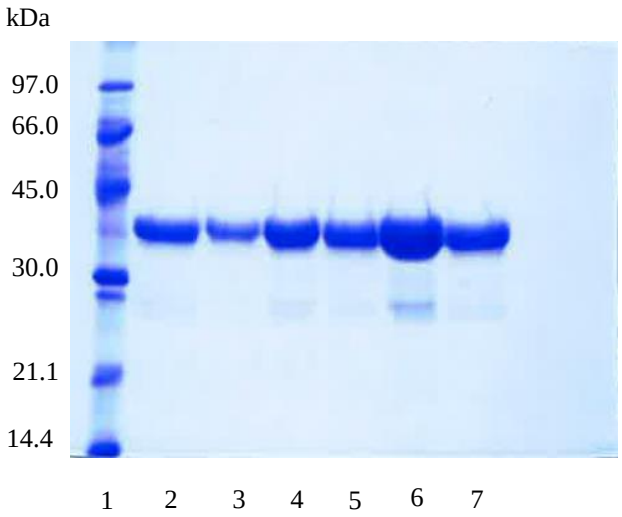

Figure 8.2. SDS-PAGE analysis of Purolite C18-TLL and Purolite C18-VS-TLL-Asp. Lane 1: Low-weight molecular markers; Lane 2: Purolite C18-TLL (0.1 g/mL rupture buffer); Lane 3: Purolite C18-VS-TLL-Asp (0.1 g/mL rupture buffer); Lane 4: Purolite C18-TLL (0.2 g/mL rupture buffer); Lane 5: Purolite C18-VS-TLL-Asp (0.2 g/mL in rupture buffer); Lane 6: Purolite C18-TLL (0.33 g/mL rupture buffer); Lane 7: Purolite C18-VS-TLL-Asp (0.33 g/mL in rupture buffer); Three biocatalyst concentrations were used to allow visual comparison of band intensity regarding to amount of protein desorbed from the supports, allowing to estimate the percentage of protein non-covalently attached; Other specifications are described in Methods.

8.3.2 Preparation of Purolite C18-EDA-VS

To improve the covalent immobilization of TLL on Purolite C18-VS, we decided to oxidize the support, the produced aldehydes were made to react with ethylenediamine, and the support was then modified with vinyl sulfone. Amino-VS supports have already reported to be advantageous in enzyme immobilization [59,60].

The elemental analysis of Purolite-C18-VS showed that the support presented around 22 micromoles of S per dried g of support, while Purolite-C18-EDA-VS presented around double the amount of S. That way, the modified support has some advantages; there are a higher number of vinyl sulfone groups, the VS reactive groups have a longer spacer arm (4 additional atoms) making reaction with the enzyme easier [25] and the ionic character of the groups under the VS layer can establish ionic bridges with the enzyme, favoring the approaching of the enzyme to the vinyl sulfone groups [69].

Figure 8.3 shows that the immobilization on this trifunctional support follows a similar pattern to the use of Purolite C18 (45% of the offered enzyme activity is immobilized, allowing to infer that around 9 mg of enzyme/g of support are immobilized). However, in this instance, after incubation and blocking, the enzyme could not be released to the medium when boiled in SDS (Figure 8.4). This suggested that we have got some enzyme-support covalent bonds for each enzyme molecule [63,70]. That way, we have been able to sort out the problems of the use of this support to prepare an interfacially activated and covalently immobilized TLL on this support.

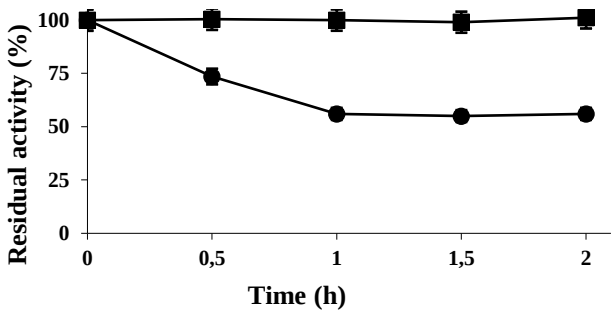

Figure 8.3. Immobilization course of TLL on Purolite C18-EDA-VS beads using an enzyme loading of 20 mg/g. The immobilization was performed in 5 mM sodium acetate at 25 °C and pH 5.0. Squares: reference and circles: supernatant. Other specifications are described in Methods.

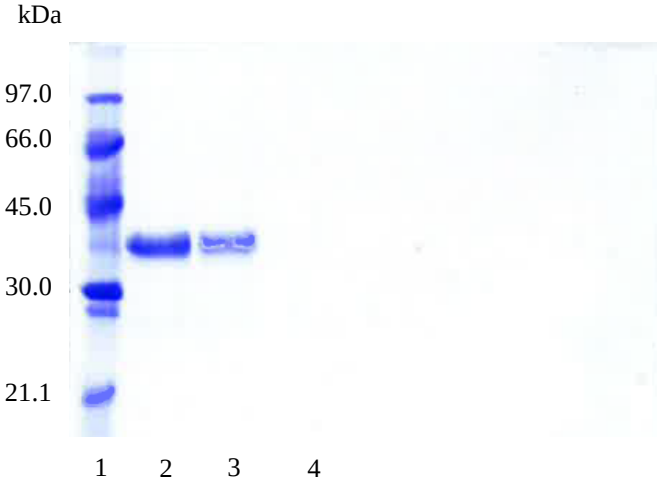

Figure 8.4. SDS-PAGE analysis of different TLL biocatalysts. Lane 1: Low-weight molecular markers; Lane 2: Purolite C18-TLL (0.1 g/mL rupture buffer); Lane 3: Purolite C18-VS-TLL-Asp (0.1 g/mL rupture buffer); Lane 4: Purolite C18-EDA-VS-TLL-Asp (0.1 g/mL rupture buffer). Other specifications are described in Methods.

8.3.3 Optimization of the Purolite C18-EDA-VS-TLL biocatalyst

It has been shown in some papers how the blocking step can greatly alter the lipase features when immobilized on supports activated with vinyl sulfone (mono or heterofunctional supports) [52–55,59,60]. That way, in this instance we blocked the remaining VS groups in the biocatalyst with 4 different compounds. Three are amino acids, a small one bearing the same number of cationic and anionic groups (Gly), another larger one but also bearing a cationic and an anionic group (Cys) and the last one bearing a cationic and two anionic groups (Asp). We also used ethylenediamine (EDA), bearing two cationic groups. It should be considered that in this instance, all biocatalysts already have EDA (even under the VS groups) and octadecyl groups, that way the effect of the modification of the remaining VS groups could be smaller than those found in other instances.

However, this was not the case. Table 8.1 shows the activities of the four Purolite C18-EDA-VS-TLL biocatalysts versus triacetin, *R*- or *S*-methyl mandelate, compared to that of Purolite C18-TLL biocatalyst. For all biocatalysts the highest activity was obtained using triacetin, and the enzyme was more active versus *R*- than versus *S*-methyl mandelate. Similar behavior was previously reported by Guimarães et al. [71] using octyl-agarose as support, but all biocatalysts had a triacetin activity at least 5-fold less than the biocatalysts prepared in Purolite (all biocatalysts had protein loads of around 10 mg/g). The most active biocatalyst with all substrates was Purolite C18-TLL, but there are large differences among the Purolite C18-

VS-TLL biocatalysts. The blocking with Cys always produced the highest decrease in enzyme activity, to 60% using triacetin, to around 70% using *R*-methyl mandelate and to 30% for the *S*-isomer. That way, the *R/S* activity ratio was increased, from 7 using Purolite C18-TLL to 17.8 using Purolite C18-EDA-VS-TLL-Cys. The most active Purolite C18-VS-TLL biocatalyst using triacetin was Purolite C18-EDA-VS-TLL-Asp shortly followed by Purolite C18-EDA-VS-TLL-Gly, being the less active among these three Purolite C18-EDA-VS-TLL-EDA (showing over 85% of the activity of the Purolite C18- TLL). However, Purolite C18-EDA-VS-TLL-EDA was the most active versus *R*-methyl mandelate, maintaining the values of Purolite C18-TLL, being the activities of the other two preparations very similar and 90% of this value. Moving the *S*-methyl mandelate, again Purolite C18-EDA-VS-TLL-EDA was the least active, and the other two biocatalysts gave similar activities (2/3 of the activity of Purolite C18- TLL). That way, Purolite C18-EDA-VS-TLL-EDA presented the highest *R/S* activity ratio (20), approximately 3-fold higher than that of the Purolite C18-TLL. This showed how the enzyme specificity can be also tuned in the blocking step using in this trifunctional support [52–55,59,60]. Here, the *R/S* activity ratio was much higher (up to around 28 times) than those of commercial immobilized lipases (Lipozyme®TL, Lipozyme®435, Lipozyme®RM and LipuraSelect) reported by Guimarães et al. [72]. Previously using octyl-agarose as support, the biocatalyst agarose C8-VS-TLL-EDA also yielded an increase of the *R/S* activity ratio of around 3-fold compared to the agarose C8-TLL, but the *R/S* activity ratio ranged from 2 to 5 [65].

Table 8.1. Mass activity of different biocatalysts with 50 mM triacetin and 50 mM *R*- or *S*-methyl mandelate in 50 mM sodium phosphate at pH 7 and 25 °C. Experiments were conducted as described in Methods.

Biocatalysts	**Activity (U/g)**		
	Triacetin	***R*-Mandelate**	***S*-Mandelate**
Purolite C18-TLL	548.7 ± 25.9	2.2 ± 0.2	0.32 ± 0.01
Purolite C18-EDA-VS-TLL-Gly	526.7 ± 20.5	2.0 ± 0.1	0.22 ± 0.01
Purolite C18-EDA-VS-TLL-EDA	479.6 ± 24.5	2.2 ± 0.1	0.11 ± 0.06
Purolite C18-EDA-VS-TLL-Asp	534.7 ± 30.0	2.0 ± 0.1	0.20 ± 0.01
Purolite C18-EDA-VS-TLL-Cys	327.5 ± 10.2	1.6 ± 0.1	0.09 ± 0.01

Next, we analyzed the effect of the blocking step on the biocatalyst's stabilities (Figure 8.5). We have included in the study the enzyme immobilized on octyl agarose, a biocatalyst considered as highly stabilized [54], to show the advantages of Purolite C18; the enzyme is more stable when immobilized on Purolite C18 (agarose-octyl-TLL retained less than 10% of the initial activity after 4 h of incubation at 75 °C and pH 7, while Purolite C18-TLL retained almost 55%). The different Purolite C18-VS-TLL biocatalysts presented very different stabilities. The most stable one was the biocatalyst blocked with Cys. This biocatalyst even showed an initial increase in its activity when incubated at high temperature and maintained next to 90% of the initial activity after 4 h of inactivation. The initial increase of enzyme activity can be promoted by some conformational changes that can produce a structure more similar to the native one, as this was the one that had lost more activity versus the 3 assayed substrates during its preparation. The biocatalysts blocked with Asp presented a thermal stability slightly under that of Purolite C18-TLL, while Purolite C18-EDA-VS-TLL-EDA and even more Purolite C18-EDA-VS-TLL-Gly were slightly more stable.

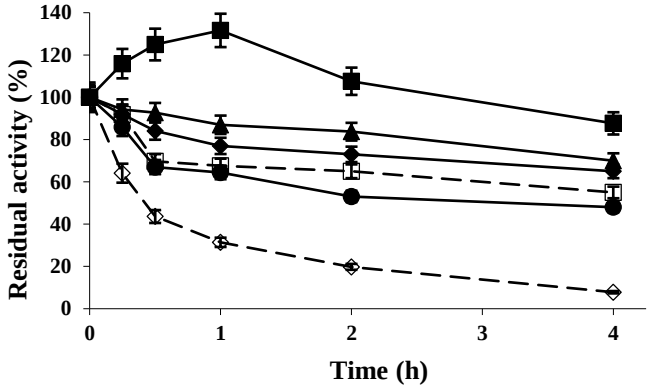

Figure 8.5. Inactivation courses of different TLL biocatalysts in 10 mM Tris-HCl buffer at pH 7.0 and 75 °C. Other specifications are described in Methods. Agarose-octyl-TLL (◇); Purolite C18-TLL (□); Purolite C18-EDA-VS-TLL-Gly (◆); Purolite C18-EDA-VS-TLL-EDA (▲); Purolite C18-EDA-VS-TLL-Asp (●); Purolite C18-EDA-VS-TLL-Cys (■).

That way, the blocking step produced biocatalysts with quite different functional features that have been previously correlated to changes in the enzyme conformation [43], even using this trifunctional supports.

8.4 Conclusions

The use of Purolite C18-EDA-VS has permitted to get an TLL biocatalyst that is first immobilized via interfacial activation and later covalent attached to the support, preventing any risk of enzyme release. The enzyme immobilized on Purolite C18 was significantly more stable than the enzyme immobilized on octyl agarose showing the great interest of this biocatalyst. The blocking step of the Purolite C18-EDA-VS biocatalyst was found to be a critical one in the preparation of the biocatalyst, as it determines not only the enzyme activity and specificity, but also enzyme stability. The selection of the optimal biocatalysts must be performed after an empiric study, as some modifications are negative for the activity versus a substrate and positive versus other.

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CHAPTER 9
IMPROVING PERFORMANCE OF *Thermomyces lanuginosus* IMMOBILIZED ON OCTADECYL-VINYL SULFONE METHACRYLATE BEADS

Thermomyces lanuginosus immobilized on Purolite@ C18 activated with vinyl sulfone was used for the production of fatty acid methyl esters (FAME). Various vegetable oils with different fatty acid composition were used in this study. The biocatalysts showed better performance in the methanolysis of linseed oil, which is rich in unsaturated fatty acids. The FAME yield was 1.9-fold lower when the reaction was performed with oil rich in saturated fatty acids. The Purolite C18-EDA-VS-TLL-EDA biocatalyst was used in an experimental design and reached a FAME yield of 74 wt.% using enzyme load of 7.5 wt.%, methanol/oil molar ratio of 3, and temperature of 35 °C after 1 h of reaction. In a vortex flow reactor (VFR), this biocatalyst reached a FAME yield of 81 wt.% after 120 min and reaction equilibrium has been reached. This result may be related to the accumulation of water in the system, limitation of the biocatalyst to convert some fatty acids present in linseed oil into methyl esters (e.g. saturated fatty acids), or deleterious effect of methanol on the biocatalyst, resulting in a decrease in the catalytic performance. However, the viability of the enzymatic approach for the production of esters was demonstrated, in addition, to obtain a product that can be used as Ecodiesel.

9.1 Introduction

Current concerns about rising global temperatures and declining oil supplies have encouraged research into biodegradable and environmentally friendly fuels. Ecofuels (e.g., biodiesel or Ecodiesel) have become attractive alternative fuel [1] and can be produced by transesterification of animal fats or vegetable oils or by direct esterification of fatty acids [2,3] with small-chain alcohols [4–7]. These reactions are performed with heterogeneous or homogenous catalysts. Heterogeneous catalysts are preferred from an industrial perspective due to their simpler recovery and low production of chemical waste [8].

In biodiesel production, mostly formed by fatty acid ethyl or methyl esters, alkaline or acid catalysis are the most used strategies, though they have some problems, such as producing unwanted secondary products and requiring a raw material with certain characteristics (low acidity, low water content) [9,10]. Immobilized lipases are good alternative heterogeneous catalysts for the synthesis of these esters as a result of low energy consumption, possibility to

use oils not purified (acidic oils can be used), and simplicity of the downstream of the product (production of by-products lower) [8,9,11].

There are a great number of techniques and supports suitable for enzyme immobilization. In the case of lipases, a popular technique that has been widely reported is their immobilization by hydrophobic adsorption on highly hydrophobic surfaces [12–15]. Lipase has a special mechanism, called interfacial activation, a process that enables the enzyme to exist in two forms in equilibrium: a closed form, in which a lid (polypeptide chain) covers the enzyme active site, and an open form, where the cover is moved allowing the lipase to adsorb on hydrophobic surfaces (e.g., drops of oils, air bubbles, etc.) [16–19] and turn the active site available for the substrate [19]. Immobilization on these supports intervenes and stabilizes the open shape of the lipase [20]. The main problem with immobilization on a hydrophobic support is the possibility of enzyme desorption when the biocatalyst is subjected to drastic conditions, such as high temperature or the presence of an organic solvent [21]. Furthermore, when applied in biosurfactant synthesis, reactions where biosurfactant is an intermediate product of the process or in heterogeneous medium containing substances with detergent properties [22].

A strategy used to minimize this problem is immobilization on a heterofunctional support. This type of support has a unique surface exhibiting various physicochemical capabilities, a layer of acyl groups to obtain the lipase interfacial activation and a layer of groups able to give other physical interactions (e.g., ionic exchange [23]) or a covalent bond that will make the immobilization irreversible [24–28]. This new biocatalysts are generally even more stable than standard biocatalysts immobilized only by hydrophobic interaction [29]. Covalent bonds were tested with glyoxyl, glutaraldehyde or vinyl sulfone groups. The latter were most successful because of their longer spacer arm and reactivity with very different nucleophilic groups on the surface of the enzyme. Furthermore, the blocking step required using this immobilization strategy gave the opportunity to generate special enzyme-support interactions, which fully altered the enzyme features [26,30–32] and even the inactivation pathway [32,33].

Recently, *Thermomyces lanuginosus* (TLL) immobilized on Purolite$^{@}$ C18 showed very good results in the production of biodiesel, being in the same order of magnitude as the reaction rate using an alkaline catalyst [34–36]. In this context, our research effort is to apply TLL immobilized on this support activated with vinyl sulfone [37], to further improve the biodiesel production. Various vegetable oils with different fatty acid composition were used in this study. The variables (methanol/oil molar ratio, enzyme load and temperature) that influence the transesterification were studied, analyzing also likely interactions between the variables [5,38,39].

9.2 Materials and Methods

9.2.1 Materials

Liquid TLL formulation with 20.8 mg protein/mL (kindly donated by Novozymes Spain (Madrid, Spain)) was utilized in this paper. Bradford´s reagent (utilized to calculate the protein concentration [40]), sodium periodate, sodium borohydride, ethylenediamine (EDA), and the amino acids glycine (Gly), aspartic acid (Asp), and cysteine (Cys) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Divinyl-sulfone (DVS) was purchased from Thermo Fisher Scientific Spain (Madrid, Spain). Methanol was purchased from Neon (São Paulo, SP, Brazil). Linseed, coconut and castor oil were purchased at the local market (São Carlos, SP, Brazil). Purolite Lifetech® ECR8806F (methacrylate macroporous resin containing octadecyl - C18 - groups) (Purolite® C18) was kindly donated from Purolite® Ltd. (Wales, UK). All other reagents were of analytical grade.

9.2.2 Methods

All experiments were performed at least by triplicate, and the values are presented as mean values and standard deviation.

9.2.2.1 Wetting of Purolite® C18 beads

Prior to use, 1 g of Purolite® C18 beads were suspended in 5 mL of methanol and kept under gentle agitation for 1 h to remove the air inside the particles [34]. Subsequently, 5 mL of distilled water was added, maintaining the stirring for 15 min. Afterwards, the suspensions were vacuum filtered, washed 10 times with 20 volumes of distilled water, and stored at 4-6 °C.

9.2.2.2 Preparation of octadecyl-vinyl sulfone Purolite® beads

The octadecyl-vinyl sulfone (VS) Purolite support was prepared following the methodology described by Guimarães et al. [37]. An amount of 200 μmol of sodium periodate per g of support was added to a suspension of Purolite® C18 beads (0.1 g/mL) prepared in distilled water to oxidize the support producing aldehyde groups. The oxidation process was monitored by spectroscopy at 450 nm (isosbestic point), adding 100 microliters of the oxidation suspension to a mixture of 1 mL of sodium bicarbonate saturated solution and 1 mL of 1 M potassium iodide. Oxidation took place under gentle agitation for 3 h at 25 °C. Then, the suspension was vacuum filtered and washed 10 times with 20 volumes of distilled water. After recovery, the support was resuspended in 2 M EDA at pH 10.0 (1:10 ratio, w/v). The suspension

was incubated with gentle agitation for 48 h. Then, solid sodium borohydride was added to reach a concentration of 10 mg/mL. The reduction was carried out under gently stirring for 30 min. Next, the suspension was vacuum filtered and washed 10 times with 20 volumes of distilled water to recover the activated support. After, 10 g of the aminated support was added to 200 mL of 350 mM divinyl-sulfone prepared in 333 mM sodium carbonate at pH 11.5 under gently stirring for 2 h. Subsequently, the support was vacuum filtered, washed 10 times with 20 volumes of distilled water, and stored at 4-6 °C.

9.2.2.3 TLL immobilization

9.2.2.3.1 Immobilization of lipases on wet Purolite® C18 beads

TLL was immobilized by interfacial activation in previously hydrated Purolite® C18 beads using an enzyme loads of 20 mg/g. An excess of enzyme was utilized to ensure that the support surface was fully coated with the enzyme. 10 g of support was added to 100 mL of enzyme solution prepared in 5 mM sodium phosphate at pH 7.0. The immobilization was conducted at room temperature under gentle stirring for 2 h, measuring the activity in supernatant and reference using *p*-NPB assay. Afterwards, the suspensions were vacuum filtered, washed 10 times with 20 volumes of distilled water, and stored at 4-6 °C.

9.2.2.3.2 Immobilization of lipases on octadecyl-vinyl sulfone Purolite® beads

10 g of support was added to 10 mL of enzyme solution prepared in 5 mM sodium acetate at pH 5.0 (enzyme loads of 20 mg/g, exceeding the maximum load capacity of the support in purpose). The pH 5.0 was used to favor interfacial activation as the main first cause for enzyme immobilization [41]. The immobilization was conducted at room temperature under gentle stirring for 2 h, measuring the activity in supernatant and reference using *p*-NPB assay. The enzyme immobilized on this support almost expressed no activity versus this substrate. Afterwards, the suspensions were vacuum filtered and washed 10 times with 20 volumes of distilled water. After recovery, 1 g of the immobilized enzyme was suspended in 10 mL of 100 mM sodium carbonate at pH 10.0. The suspension was incubated at room temperature for 24 h to favor the formation of covalent bonds between enzyme-support. Afterwards, the biocatalysts were vacuum filtered. After recovery, 1 g of biocatalyst was added to 10 mL of 2 M blocking agent (Gly, Asp, Cys, or EDA) at pH 10.0. The modification of remaining vinyl sulfone was carried out at 25 °C under gentle agitation for 48 h. Subsequently, the support was vacuum filtered, washed 10 times with 20 volumes of distilled water, and stored at 4-6 °C.

9.2.2.4 Enzyme activity assays

One unit of activity (U) was defined as the amount of enzyme that hydrolyzes one μmol of substrate per minute under the described conditions.

9.2.2.4.1 Hydrolysis of p-NPB

50 μL of a soluble enzyme solution or supernatant of enzyme suspension (e.g., in enzyme immobilization suspensions) was added to a mixture of 50 mL of 10 mM *p*-NPB prepared in acetonitrile and 2.5 mL of 25 mM sodium phosphate at pH 7.0. The hydrolysis was conducted using a thermostatization system at 25 °C under magnetic stirring for 1.5 min. The *p*-nitrophenol released into the medium was monitored by spectrophotometry at 348 nm (isosbestic point) to determine the hydrolytic activity ($\varepsilon = 5150\ M^{-1}\ cm^{-1}$) [42].

9.2.2.5 Methanolysis of different oils

The methanolysis of different oils (linseed, coconut, and castor oil) were evaluated with different biocatalysts. The reactions were carried out in sealed glass vials (100 mL capacity) using 2 g of oil, methanol/oil molar ratio of 3, enzyme load of 5 wt.%, temperature of 40 °C under agitation at 250 rpm for 3 h. After this period, aliquots were collected to quantify the FAME yield by gas chromatography.

9.2.2.6 Experimental design

The effects of temperature (31.6 – 48.4 °C), methanol/ oil molar ratio (0.96 – 11.04) and enzyme load (0.8 – 9.2 wt.%) under the transesterification reaction were studied using a central rotational composite design (2^3 with three central points and six axial points), as shown in Table 9.1. The reactions were carried out in sealed glass vials (100 mL capacity) using 2 g of refined linseed oil under agitation at 250 rpm for 1 h. After this period, aliquots were collected to quantify the FAME yield by gas chromatography.

Table 9.1. Levels of independent factors for Central Composite design.

Variables		-1.68	-1	0	+1	+1.68
Methanol/oil molar ratio	X_1	1	3	6	9	11
Enzyme load, wt.%	X_2	0.8	2.5	5	7.5	9.2
Temperature, °C	X_3	31.6	35	40	45	48.4

The analysis of the results was performed in the Statistica software (StatSoft, version 7.0) and a mathematical model was generated to predict the effect of the selected factors on the FAME yield (Equation 1):

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ij} X_i X_i \quad (1)$$

where y is the predicted response variable; β_0, β_i, β_{ii}, β_{ij} are the intercept, linear, quadratic and interaction constant coefficients of the model, respectively; X_i, X_j ($i = 1, 3$; $j = 1, 3$; $i \neq j$) represent the coded independent variables (reaction conditions).

9.2.2.7 Transesterification of linseed oil with hydrated ethanol

After validation of the experimental data, a transesterification reaction of the linseed oil with methanol was carried out under the optimized conditions in the experimental design. The reaction was carried out in a vortex flow reactor (VFR) operated under agitation at 2000–2500 rpm. Aliquots were taken to analyze FAME yield by gas chromatography.

9.2.2.8 Gas Chromatography Analysis

FAME were analyzed in accordance with ASTM D6751 and EN14103 [43], modified by [6]. For this purpose, a 7890A Agilent chromatograph (Santa Clara, CA, USA) equipped with Rtx-Wax capillary column (30 m × 0.25 mm × 0.25 μm, Restek Corporation, Bellefonte, PA, USA) and a flame ionization (FID) detector was used. The column was set at 210 °C and detector and split/splitless injector (split ratio of 1:50) at 250 °C. Helium was used as carrier gas (0.5 mL min^{-1}), methyl heptadecanoate as an internal standard, split ratio of 1:50 and 25 min analysis time. Samples were prepared by dissolution of 50 mg of the washed and dried sample into 1 mL of internal standard solution (10 mg mL^{-1}, in heptane) and 1 μL was applied to the equipment. The ester mass yield (wt.%) was calculated in accordance with Equation 2:

$$\text{Ester yield (wt.\%)} = \frac{(\sum A) - A_{SI}}{A_{SI}} \times \frac{C_{SI} \times V_{SI}}{m} \times 100 \quad (2)$$

where C_{SI} corresponds to concentration of the internal standard (10 mg mL^{-1}), A_{SI} corresponds to peak area of the internal standard (C17), ΣA is the total peak area of fatty acid ethyl esters

C14:0 to C24:0, V_{SI} and m corresponds to volume of the internal standard solution (1 mL) and mass of sample (50 mg), respectively.

9.3 Results and Discussion

9.3.1 Evaluation of biocatalysts in the methanolysis of different vegetable oils

In this study, three oils with different fatty acid composition were used. Coconut oil has about 86 wt.% of saturated fatty acids and 7 wt.% of unsaturated fatty acids, mainly composed of lauric acid (44 wt.%). Linseed oil has 11 wt.% of saturated fatty acids and 89 wt.% of unsaturated fatty acids, mainly composed of linolenic acid (54 wt.%). Castor oil has 5 wt.% of saturated fatty acids and 93 wt.% of unsaturated fatty acids, mainly composed of ricinoleic acid (88 wt.%) which has a hydroxyl group on carbon 12 [44]. As shown in Table 9.2, the behavior of TLL immobilized by interfacial adsorption (Purolite C18-TLL), TLL immobilized by interfacial adsorption followed by covalent (Purolite C18-EDA-VS-TLL-blocking agent), and commercially immobilized TLL showed different behavior in the methanolysis of the different oils. Thus, it was demonstrated on the power of immobilization to tune enzyme functional features, including examples where the only difference is the final physical interactions between enzyme and support (i.e., octyl-vinyl sulfone biocatalysts blocked with different reagents). Purolite C18-TLL and Purolite C18-EDA-VS-TLL-EDA biocatalysts showed better adjustment for methanolysis of the different oils, however, the highest yield was using linseed oil. The FAME yield was 1.4-fold lower when the reaction was performed with oil rich in saturated fatty acids. Furthermore, FAME yield was 1.9-fold lower when the reaction was performed with oil rich in ricinoleic acids, possibly there is a steric effect due to the presence of the hydroxyl group in this fatty acid. Commercially immobilized TLL underperformed in all cases.

9.3.2 Experimental design for methanolysis of linseed oil

The range of values for each variable in the experimental design was defined based on studies in the literature for fatty acid methyl esters production [5,38,39,45]. Table 9.3 describes the coded and real levels, as well as the experimental results. The results showed that the maximum yield of FAME was about 74 wt.% after 1 h of reaction (Run 3).

Table 9.2. Methanolysis of different oils to produce fatty acid methyl esters (FAME). Conditions: Methanol/oil molar ratio of 3, enzyme load of 5 wt.%, temperature of 40 °C, and stirring at 250 rpm for 3 h.

Biocatalysts	FAME yield, wt.%		
	Coconut oil	Linseed oil	Castor oil
Purolite C18-TLL	43.9 ± 3.3	62.3 ± 8.0	33.3 ± 1.8
Purolite C18-EDA-VS-TLL-Gly	38.9 ± 1.1	9.7 ± 1.0	29.9 ± 1.4
Purolite C18-EDA-VS-TLL-EDA	46.0 ± 0.3	63.1 ± 2.4	32.3 ± 0.6
Purolite C18-EDA-VS-TLL-Asp	37.9 ± 0.8	53.0 ± 0.6	30.3 ± 0.7
Purolite C18-EDA-VS-TLL-Cys	37.9 ± 0.8	53.3 ± 0.6	28.9 ± 0.4
TLL-IM	6.9 ± 0.5	13.8 ± 0.2	8.3 ± 0.3

Table 9.3. Experimental design of linseed oil methanolysis. Assay conditions: 2 g of linseed oil and stirring at 250 rpm for 1 h.

Run	Factors (Codified and actual levels)			FAME yield, wt.%
	Temperature, ºC (X_1)	Molar ratio methanol/oil (X_2)	Enzyme load, wt.% (X_3)	
1	-1 (35)	-1 (3)	-1 (2.5)	21.4
2	-1 (35)	+1 (9)	-1 (2.5)	4.4
3	**-1 (35)**	**-1 (3)**	**+1 (7.5)**	**74.1**
4	-1 (35)	+1 (9)	+1 (7.5)	27.5
5	+1 (45)	-1 (3)	-1 (2.5)	6.1
6	+1 (45)	+1 (9)	-1 (2.5)	3.06
7	+1 (45)	-1 (3)	+1 (7.5)	66.2
8	+1 (45)	+1 (9)	+1 (7.5)	11.6
9	0 (40)	-1.68 (0.96)	0 (5)	23.7
10	0 (40)	+1.68 (11.04)	0 (5)	6.8
11	0 (40)	0 (6)	-1.68 (0.8)	1.2
12	0 (40)	0 (6)	+1.68 (9.2)	47.7
13	-1.68 (31.6)	0 (6)	0 (5)	39.2
14	+1.68 (48.4)	0 (6)	0 (5)	8.0
15	0 (40)	0 (6)	0 (5)	9.2
16	0 (40)	0 (6)	0 (5)	10.6
17	0 (40)	0 (6)	0 (5)	9.52

Table 9.3 shows the statistically significant effects ($p < 0.05$) and the adequacy of the model to the FAME yields at the 95% confidence level. There was a statistically significant lack of adjustment. However, the coefficient of determination indicates that the proposed model may account for approximately 92% of the experimental variability, a satisfactory representation of the model for the linseed oil metholysis response. All linear and quadratic parameters of the studied variables were significant, as confirmed by the Pareto Diagram (Figure 9.1). Thus, the mathematical model (Equation 3) with the coded significant variables can be used to represent the FAME yields for the conditions studied:

$$\text{FAME yield (wt.\%)} = 9.5 - 6.8X_1 + 6.0X_1^2 - 11.0X_2 + 3.0X_2^2 + 16.3X_3 + 6.3X_3^2 - 10.1X_{23} \quad (3)$$

Table 9.3. Analysis of variance (ANOVA) to evaluate the factors that influence the FAME yield in the transesterification reaction of linseed oil using Purolite C18-EDA-VS-TLL-EDA.

Factors	Sum of squares	Degree of freedom	Medium square	F	*p*-valor
(1) Temperature, °C (L)	**630.2**	**1**	**630.1**	**1259.8**	**0.0008**
Temperature, °C (Q)	**405.3**	**1**	**405.3**	**810.2**	**0.001**
(2) Molar ratio methanol/oil (L)	**1643.6**	**1**	**1643.6**	**3285.8**	**0.0003**
Molar ratio methanol/oil (Q)	**104.7**	**1**	**104.7**	**209.4**	**0.005**
(3) Enzyme load, wt.% (L)	**3625.3**	**1**	**3625.3**	**7247.6**	**0.0001**
Enzyme load, wt.% (Q)	**446.9**	**1**	**446.9**	**893.4**	**0.001**
2L*3L	**824.0**	**1**	**824.0**	**1647.4**	**0.0006**
Lack of fit	**607.2**	**7**	**86.8**	**173.4**	**0.006**
Pure error	1.0	2	0.5		
Total square sum	7990.3	16			

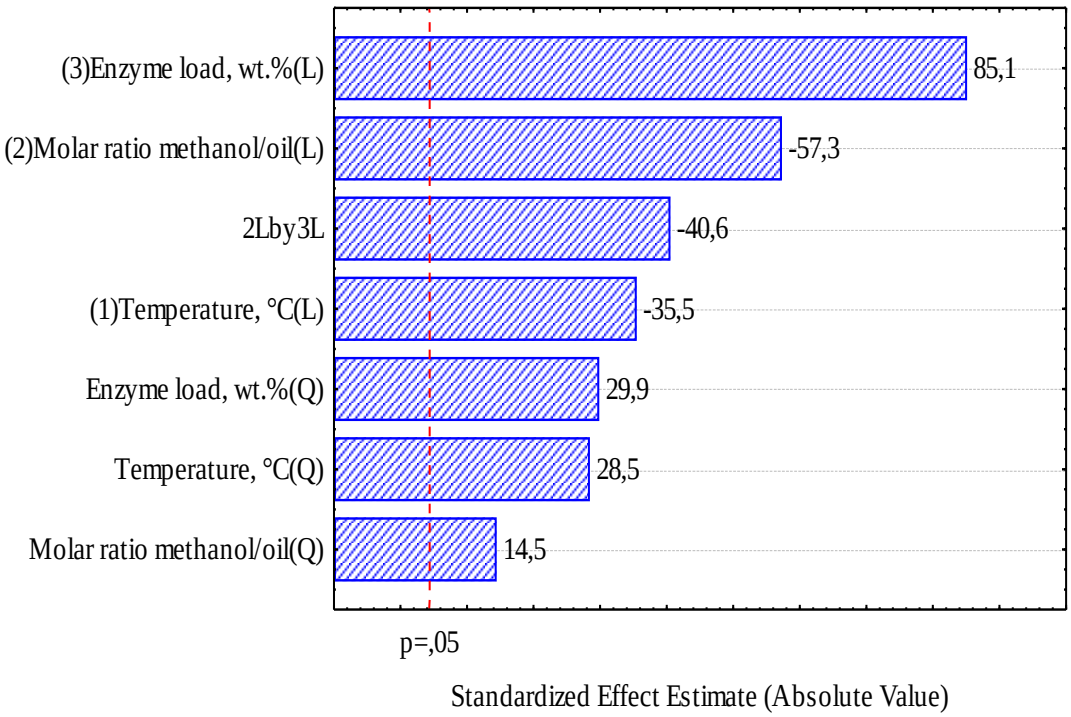

Figure 9.1. Pareto Diagram for the estimated effect of each planning variable.

Figure 9.2 shows the response surfaces constructed from the mathematical model and it can be seen that all variables influence the FAME yield. There are ranges of temperature values that maximize the methanolysis of linseed oil, however, there is a direct dependence on the enzyme load and the methanol/oil molar ratio (Figures 9.2a and 9.2b). While the increase of the enzyme load leads to the increase of the FAME yield when the methanol/oil molar ratio is reduced (Figure 9.2c). This demonstrates that methanol has a deleterious effect on the biocatalyst, as has been demonstrated in the literature [11].

Figure 9.3 shows the regions of optimized values of the three experimental parameters for the maximum yield in FAME. The red dotted lines correspond to the optimal values for each variable: methanol/oil molar ratio of 6.0, enzyme load of 9.2 wt.% and temperature of 31.6 ºC. For these conditions, the maximum expected FAME yield is 83.0 wt.%.

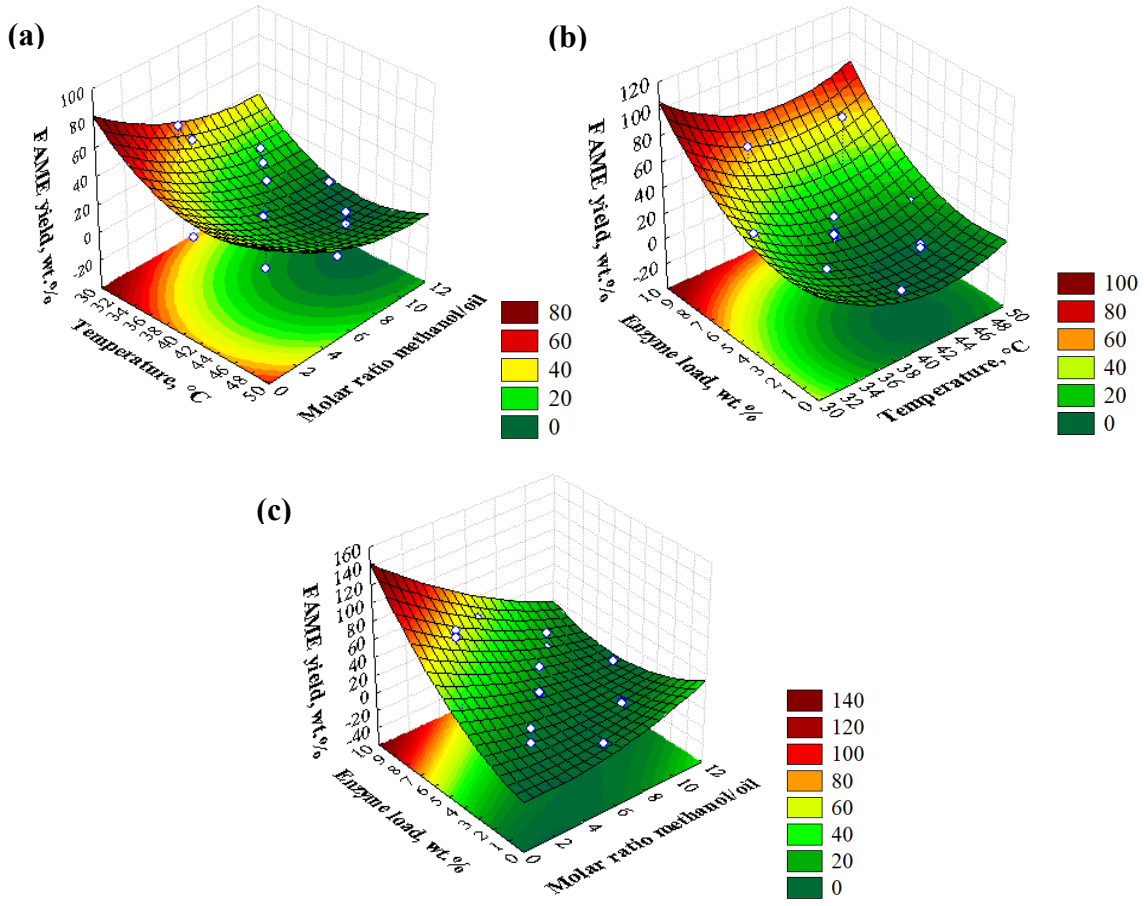

Figure 9.2. Response surfaces for the effects of independent variables on FAME yield. (a) Temperature and methanol/oil molar ratio, (b) enzyme load and temperature, and (c) enzyme load and methanol/oil molar ratio.

9.3.3 Experimental validation of the linseed oil methanolysis model

The validation of the model was carried out in a vortex flow reactor (VFR) using an enzyme load of 9.2 wt.%, temperature of 35 °C and methanol/oil molar ratio of 3, as shown in Figure 9.4. Purolite-EDA-VS-TLL-EDA showed a high initial rate of transesterification in the initial moments and reached maximum FAME yield (81 wt.%) in 120 min of reaction. Equilibrium was reached and no change in FAME yield was observed. The maximum theoretical FAME yield obtained by the equation is 98 wt.%, while the experimental value was 81 wt.%. This demonstrates an experimental deviation of 17% that may be related to the accumulation of water in the system, limitation of the biocatalyst to convert some fatty acids present in linseed oil into methyl esters (e.g. saturated fatty acids comprising about 12 wt.%), or deleterious effect of methanol on the biocatalyst, resulting in a decrease in the catalytic performance. An alternative to shift the equilibrium towards synthesis is to perform the transesterification of linseed oil operating with methanol-fed batch.

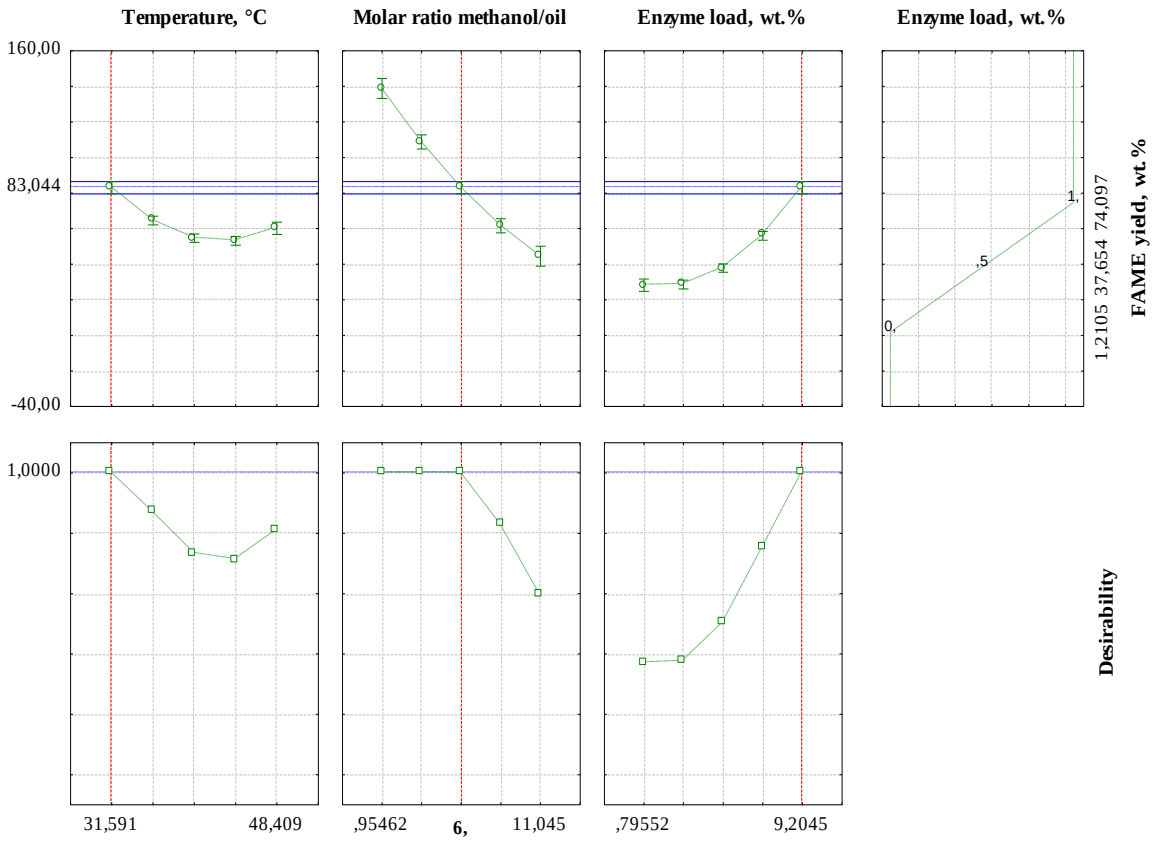

Figure 9.3. Profile of predicted/optimized values and desirability function for experimental design.

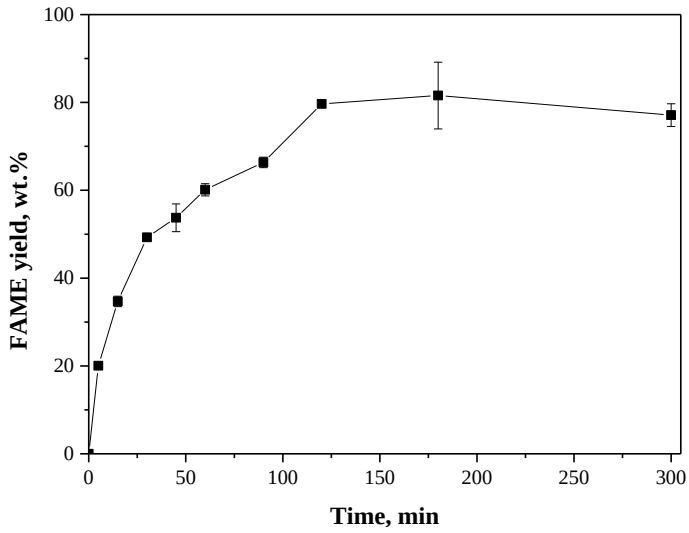

Figure 9.4. Time course of methanolysis of linseed oil using the Purolite-EDA-VS-TLL-EDA. Conditions: Reaction carried out in a Vortex Flow Reactor operated with a methanol/oil molar ratio of 3, temperature of 35 °C, 9.2 wt.% and agitation of 2000-2500 rpm.

9.4 Conclusions

The use of TLL immobilized on aminated resin and activated with vinyl sulfone groups was reported for the first time in the methanolysis of linseed, coconut, and castor oil. The best performance was observed for linseed oil, which is rich in unsaturated fatty acids. The Purolite-EDA-VS-TLL-EDA biocatalyst presented a FAME yield of 81 wt.%, while the immobilized commercial TLL had a lower performance. Therefore, the viability of the enzymatic approach for the production of esters was demonstrated, in addition, to obtain a product that can be used as Ecodiesel.

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CHAPTER 10
A TWO-STEP HYDROESTERIFICATION REACTION SYSTEM TO PRODUCE OCTYL ESTERS USING DEGUMMED SOYBEAN OIL AS SUBSTRATE: COMBINING REUSABLE FREE AND IMMOBILIZED LIPASES

The present study shows the results of enzymatic synthesis of octyl alcohol-bases biolubricants carried out in two steps: degummed soybean oil (DSO) hydrolysis and esterification of produced free fatty acids (FFA). *Pseudomonas fluorescens* free lipase (PFL) was the best lipase as catalyst in the hydrolysis reaction (97% yield in 23 h using 50 U/g oil and DSO:water mass ratio of 1:0.5). Sequential batch strategies used for the recovery and reuse of PFL have been developed. When hexane was used in the separation between the FFA-rich phase and the phase composed of water, enzyme and glycerol, there was better PFL recovery and FFA-conversion of 65% was achieved after five 24 h-cycles. In the esterification step, the potential of three lipases (lipase B from *Candida antarctica* (CALB), lipase from porcine pancreas (PPL) and Eversa® Transform 2.0 (EV)) immobilized on Purolite Lifetech EC8806F to produce octyl esters in a solvent-free medium was evaluated. The immobilized EV showed better yield in ester synthesis (yield in octyl esters ~87 wt.%) compared to other enzymes, including Lipozym-435. When EV-Purolite was used in a vortex flow reactor, maximum yield of around 94 wt.% was reached after 3 h of reaction using biocatalyst load of 1% (w/v) and FFA/octyl molar ratio of 1:2.5. The immobilized lipase could be reused in five 3 h-batches of esterification, maintaining the octyl esters yield of the first batch. The nature of the product was confirmed by Fourier Transform infrared spectroscopy operating with attenuated total reflection (ATR-FTIR).

10.1 Introduction

Fatty acid alkyl esters (FAAE) having 22 to 26 carbon atoms are promising substitutes for mineral oil lubricants [1] due to their physico-chemical properties, such as high viscosity index, high lubrication, high flash point, low volatility, good wear performance, high thermal oxidative stability and good performance at low temperatures [1–4].

FAAE can be obtained by direct esterification of fatty acids [5,6], transesterification between fatty acid monoalkyl esters or target glycerides and the desired alcohol [7–9], or hydroesterification, a sequential process of oil/fat hydrolysis followed by esterification of purified fatty acids [1,10]. Industrially, alkaline transesterification can achieve high FAAE

yields, however, this performance is achieved with the use of feedstocks containing low concentration of free fatty acids (FFA) and water (preferably 0.5 wt.% of FFA and 400 ppm of water) to avoid formation of soap. On the other hand, in the hydroesterification reaction, the feedstocks does not require high purity [11–13] and this represents a crucial importance for the process, considering that the inputs correspond to up to 75% of the total biodiesel production cost [14]. In addition, the glycerol produced in the hydrolysis step is a by-product with higher purity [15,16].

Synthetic biolubricants can be produced using either homogeneous or heterogeneous biocatalysts. Alkaline catalysts are usually the preferential industrial choice [17]. On the other hand, lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) may be alternative catalysts due to the great number of reported processes in which they have been used, not only at lab scale [18,19] but also at industrial level. The use of these biocatalysts has several advantages for industrial processes, such as possibility of using feedstocks containing a high percentage of free fatty acid or water, low energy demand, and easy recovery and purification of the product by the lack of side products thanks to the enzyme selectivity [20–22].

The hydroesterification route for biolubricant synthesis can become an ecofriendly process by using lipases as catalysts in one or both reaction steps. In the triglyceride hydrolysis step, lipases have been used in their soluble or immobilized formulation (Table 10.1). As the cost of immobilization has been reported to be competitive with the cost of the enzyme itself [23], the use of free lipases can maximize the technical and economic viability of the process, and more if it may be coupled the enzyme reuse. Although the use of immobilized enzymes may have some advantages also in this hydrolysis process, we have decided try free lipases in this approach, developing strategies that may permit the recovering and reuse in several sequential batches. On the other hand, in the esterification step, the use of the immobilized enzyme has clearer advantages, even when there are some lipase formulations launched to be used in the production of esters of free fatty acids [24]. Together to improve the enzyme stability in the presence of alcohols and fatty acids, it avoids enzyme aggregation and consequently the loss of its activities due to the presence of solvent [25].

There are a great number of techniques and supports suitable for enzyme immobilization. In general, some criteria are followed in their choice regarding to high activity of the immobilized enzyme, high stability against temperature and organic solvents, low cost of immobilization, and low toxicity of the immobilization reagents and supports [22,39–41].

Table 10.1. Review distribution of different reaction conditions for enzymatic hydrolysis.

Lipase	Biocatalyst	Reaction conditions			Conversion (%)	Reference
		Substrate	Oil/water	Time (h)		
A. oryzae	Crude formulation	Castor oil	1:1 (w/w)	72	70	[26]
Jatropha curcas	Crude formulation	Refined palm oil; Crude palm oil; Olive oi;l Physic nut oil; Castor oil; Biodiesel waste Animal tallow	10% (w/v)	2	>27	[27]
C. rugosa	Crude formulation	Waste cooking oil	1:1 (v/v)	10	100	[28]
C. rugosa	Crude formulation	Crude palm oil	1:1 (v/v)	4	100	[29]
Y. lipolytica	Immobilized	Soybean oil	2 g oil/1.2 g water	48	92.5	[30]
Ricinus communis	Crude formulation	Canola oil	30% (w/w)	2	100	[31]
C. rugosa	Crude formulation	Soybean oil Palm oil	2:1 (w/w)	24	92 89	[32]
C. rugosa	Crude formulation	Waste cooking oil	1:4 (v/v)	30	92	[1]
Candida sp. 99–125	Immobilized	Waste cooking oil	5:6 (w/w)	15	99	[33]
Ricinus communis	Crude formulation	Macauba oil	50% (v/v)	6	99.6	[34]
Crude castor seed enzyme extract	Crude formulation	Unrefined macaw palm oil	35% (w/w)	1.83	100	[35]
Castor seeds	Crude formulation	Castor oil	1:5 (w/v)	1	93	[36]
34MDP	Immobilized	Soybean oil	50% (v/v)	4	94	[37]
Eversa[@] Transform	Crude formulation	Gac oil	1:12.79	8.41	94	[38]
Thermomyces lanuginousus	Immobilized	Waste cooking oil	1:5 (w/v)	4	-	[10]
Geotrichum candidum	Crude formulation	Waste cooking oil	44.1% (w/w)	1.33	100	[13]
Candida sp. 99e125	Crude formulation	Waste cooking oil	4:4:8 (w/w)	72	90	[3]

In the case of lipases, a popular technique that has been widely reported is their immobilization by hydrophobic adsorption on highly hydrophobic surfaces [40,42–44]. This is because the lipases have a peculiar mechanism, called interfacial activation, a phenomenon that allows the enzyme to exist in two forms in equilibrium: a closed form, in which a lid (polypeptide chain) covers the enzyme active site, and an open form, in which the lid is moved away allowing the lipase to adsorb to hydrophobic surfaces (e.g., drops of oils, air bubbles, etc.) [45–48] and turning the active site accessible to the substrate [48]. This immobilization strategy is simple procedure and allows for one-step immobilization/purification/stabilization and even

hyperactivation of lipases. Furthermore, the support can be recovered and reused after enzyme inactivation [40,44]. The set of benefits of immobilization on hydrophobic support make this technique the prime candidate to maintain its supremacy in this area. Here, the immobilization of lipases on Purolite Lifetech™ ECR8806F (methacrylate macroporous resin containing octadecyl - C18 - groups) (Purolite C18) is reported. Similar supports were used by Tacias-Pascacio with very good results to produce biocatalysts useful for the biodiesel production [49–51].

In this study, the purpose is to obtain octyl esters through hydroesterification in a two-step enzymatic process: enzymatic hydrolysis of degummed soybean oil (DSO) using a free enzyme that will be recovered and reused, and subsequent esterification of the free fatty acids (FFA) with octyl alcohol. Thus, the variables (screening of lipase, DSO/water mass ratio and enzyme load) that influence the hydrolysis process using soluble lipase were studied. Sequential batch strategies used for the recovery and reuse of free lipase have been developed. In this case, the phase rich in FFA (top phase or light phase) and the phase composed of water, enzyme and glycerol (bottom phase or heavy phase) were obtained by the decantation process carried out naturally or with the aid of organic solvent. The FFA-rich phase was esterified using octyl alcohol as acyl-acceptor. Eversa@ Transform (ET) [24], lipase B from *Candida antarctica* (CALB) [52] and lipase from porcine pancreas type II (PPL) [53] immobilized on Purolite Lifetech EC8806F were used in this step. The octyl ester was characterized by ATR-FTIR.

10.2 Materials and Methods

10.2.1 Materials

Lipase from porcine pancreas type II (PPL; powder formulation with 50980.98 ± 100.32 TBU g^{-1}), lipase from *Pseudomonas fluorescens* (PFL, powder formulation with 4632.98 ± 10.17 TBU g^{-1}), lipase from *Thermomyces lanuginosus* (TLL, liquid formulation with 47730.91 ± 2471.35 TBU mL^{-1}), lipase B from *Candida antarctica* (CALB, liquid formulation with 8274.07 ± 349.95 TBU mL^{-1}), Eversa® Transform 2.0 (EV, liquid formulation with 106871.52 ± 4575.60 TBU mL^{-1}), Bradford´s reagent, and tributyrin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purolite Lifetech™ ECR8806F (methacrylate macroporous resin containing octadecyl - C18 - groups) (Purolite C18) were kindly donated from Purolite Ltd. (Wales, UK). Octyl alcohol were purchased from Neon (São Paulo, SP, Brazil). Degummed soybean oil was from Cocamar, Maringá, PR, Brazil.

10.2.2 Methods

All experiments were performed in duplicate. The results were expressed as an average ± standard deviation (σ).

10.2.2.1 Characterization of the degummed soybean oil (DSO)

DSO was characterized physically-chemically in terms of acidity index (AOCS Ca 5A-40, 1990), iodine index (AOCS Cd 1-25, 1990), saponification index (AOCS Cd 3-25, 1990), density (AOCS Cc 10A-25, 1990) and humidity (AOCS Ca 2B-38, 2004). Viscosity was analyzed using a rheometer (Brookfield DV-III Ultra with TC-650 bath, Brookfield Brazil, Middleboro, MA, USA) and SC4-27 spindle, at temperatures of 40 °C.

10.2.2.2 Standard activity assay

The lipase hydrolytic activity on tributyrin was measured according to the procedure reported by Beisson et al. [54]. Briefly, 15, 25 and 50 mg of immobilized enzyme were added into a mixture of 1.5 mL of tributyrin, 6.0 mL of 100 mM sodium phosphate buffer (pH 7.5) and 16.5 mL of distilled water. The hydrolysis was carried out at 37 °C, stirred at 500 rpm for 5 min, and monitored in a Titrino 907 titrator (Metrohm, Herisau, Switzerland) using a 20 mM KOH solution to keep the pH of reaction at 7.5. The lipase hydrolytic activity was measured considering the consumption of KOH to neutralize the butyric acid released in the reaction medium. One tributyrin unit (TBU) was defined as the amount of enzyme necessary to release 1 μmol of butyric acid per minute under the conditions described.

The hydrolytic activity in olive oil was measured according to Soares et al. [55]. Briefly, 2.5, 5.0 and 10.0 g of enzyme were added to a mixture of 9 mL of the emulsion prepared with 30 g of olive oil and 2.1 g of gum arabic solution (7% w/v in relation to water, 48 mL of the buffer solution and 30 mL of distilled water). The hydrolytic reaction was carried out at 37 °C, stirred at 250 rpm for 5 min. At the end, 10 mL of ethanol were added to stop the reaction and 1 mL of phenolphthalein (1%, w/v) was added as titrating indicator. The hydrolytic activity was calculated considering the consumption of KOH (20 mM) to neutralize the fatty acid released in the reaction medium. The activity can be determined by Equation 1. One unit (U) was defined as the amount of enzyme needed to release 1 μmol of fatty acid per minute under the conditions described.

$$U\left(\frac{\mu mol}{g \times min}\right) = \frac{(V_f - V_i) \times 10^3 \times MW_{KOH}}{t \times w_{enzyme}} \quad (1)$$

where V_f is the volume of base consumed on titrating the sample after the reaction, V_i is the volume of base consumed on titrating the sample before the reaction, MW_{KOH} is the molecular mass of the base, t is the reaction time, and w_{enzyme} is the mass of enzyme used in the reaction.

10.2.2.3 Protein assay

Protein concentration was determined by Bradford's method [56], using bovine serum albumin (BSA) as standard protein.

10.2.2.4 Immobilization of lipases

10.2.2.4.1 Wetting of the Purolite supports

Prior to use, Purolite supports were submitted to a treatment to remove air and fill the pores with water. The procedure followed to the previously described methodology by Tacias-Pascacio et al. [57]. Briefly, the support was suspended in methanol at a 1:5 (w/v) ratio and incubated for 1 h under mild stirring; subsequently, distilled water was added to have a 50% water solution. After 15 minutes of mild stirring, the supports were filtered under vacuum and washed with 5 volumes of water (procedure performed 5 times).

10.2.2.4.2 Immobilization of lipases on wet Purolite supports

The immobilization of EV, CALB and PPL in Purolite was carried out according to the previously described methodology by Tacias-Pascacio et al. [57]. Briefly, immobilization of lipases was performed suspending 20 mg of proteins in 10 mL of sodium phosphate buffer (5 mM and pH 7), followed by the addition of 1 g of support. The mixture was incubated at 25 °C (for EV and CALB) and 5 °C (for PPL) with gently shaking. The protein concentration in the supernatant was monitored throughout the immobilization, while the enzymatic activity (expressed in TBU) was quantified at the beginning and at the end of the immobilization. When immobilization ended, the suspension was filtered and the immobilized enzymes were washed several times with distilled water, quantified the enzymatic activity (expressed in TBU), and stored at 4 °C. The parameters of immobilization yield (IY) and recovered activity (RA) were calculated according to Equations:

$$\text{IY } (\%) = \left(1 - \frac{\text{Supernatant activity at the reaction end}}{\text{Control enzyme solution activity at the reaction end}}\right) \times 100 \qquad (1)$$

$$\text{RA (\%)} = \left(\frac{\text{Immobilized enzyme activity}}{\text{Theoretical derivative activity}}\right) \times 100 \quad (2)$$

10.2.2.5 Optimized enzymatic hydrolysis to produce FFA

The screening of the lipases was carried out using DSO hydrolysis in oil/water mass ratio of 1:4 and enzyme load of 25 U/g oil. The reactions were carried out in closed glass bottles at 35 °C and stirring at 250 rpm for 5 min. After screening the lipase, the effect of several oil/water mass ratios (1:0.06, 1:0.25, 1:0.5, 1:1, 1:2, and 1:4) and enzyme load (50 and 250 U/g oil) were investigated to maximize the hydrolysis of DSO. In this set of experiments, the conditions used were the same as previously mentioned with time of 24 h. The kinetic profile of free PFL was studied in batch using a reactor with mechanical agitation. For this reaction the oil/water mass ratio of 1:0.5, enzyme load of 50 U/g oil, temperature of 35 °C and agitation of 900 rpm were used. At the end of each the reaction, aliquots of the light phase were removed, ethanol was added to the reaction medium to stop the reaction and FFA was quantified by titration using KOH. The conversion was calculated according to Equation 3 and 4, where C_{KOH} is the concentration of the base and w_{oil} is the mass oil used in the reaction.

$$\text{Acidity index (\%)} = \frac{[V_f - V_i] \times C_{KOH} \times MW_{KOH}}{w_{oil}} \quad (3)$$

$$\text{Conversion (\%)} = \frac{\text{Acidity index}}{\text{Saponification index}} \times 100 \quad (4)$$

10.2.2.6 Reuse of free enzyme

The free PFL was recycled in sequential batches. The addition presence and absence of hexane was used for heavy phase recovery. After 24 h of reaction, the reaction medium went through two processes: 1) it was left to rest for 3.5 h or 2) hexane was added to an FFA/hexane mass ratio of 1:1, followed by rest for 30 min. The light phase (composed of FFA and non-hydrolyzed acylglycerols) was removed, while the heavy phase (composed of water, enzyme and glycerol) was used in a new batch of reaction. In this case, the oil/water mass ratio was the same used in the first batch.

10.2.2.7 Enzymatic esterification to produce octyl esters

FFA produced in the hydrolysis step were washed twice with distilled water and separated from glycerol by centrifugation. Afterwards, it was dried in an oven at 70 °C for 24 h and used in the esterification reaction. Octanol was used as acyl acceptor in the FFA/octanol molar ratio of 1:2.5 [58]. EV, CALB and PPL immobilized on Purolite were used as biocatalysts (1%, w/v). The esterification reactions for screening the biocatalyst were carried out in screw-capped glass bottles with 50 mL of capacity containing 5 mL of reaction mixture, at 37 °C, 250 rpm for 24 h. While, the reaction to evaluate the performance of the biocatalyst were performed at 37 °C in a vortex flow reactor with rotation of the inner cylinder (metallic rod coupled to an IKA overhead stirrer, IKA Werke GmbH & Co. KG, Breisgau, Germany) at 1700–2000 rpm stirring [59]. The radius ratio ($\eta = R_{in}/R_{out}$) and aspect ratio ($\Gamma = L/d$) were 0.24 and 6.72, respectively. Samples were withdrawn to analyze octyl fatty acid esters by gas chromatography (Section 2.2.9.1).

10.2.2.8 Operational stability of EV-Purolite in the esterification reaction

EV-Purolite biocatalyst was utilized in successive 3 h-batches of esterification of DSO with octyl alcohol at 37 °C in a vortex flow reactor at 1700–2000 rpm stirring. The reaction mixture contained DSO, octyl alcohol to an octyl/DSO molar ratio of 2.5, and enzyme load of 1% (w/v). After each 3 h-cycle, the biocatalyst was recovered by centrifugation at 5,000 rpm for 5 min. At the end of each batch, octyl esters was analyzed by gas chromatography.

10.2.2.9 Characterization of the reaction products

10.2.2.9.1 Gas Chromatography Analysis

Octyl esters were analyzed in accordance with ASTM D6751 and EN14103 [60], modified by [61]. For this purpose, a 7890A Agilent chromatograph (Santa Clara, CA, USA) equipped with Rtx-Wax capillary column (30 m × 0.25 mm × 0.25 μm, Restek Corporation, Bellefonte, PA, USA) and FID detector was used. The column was set at 210 °C and detector and split/splitless injector (split ratio of 1:50) at 250 °C. Helium was used as carrier gas (0.5 mL min^{-1}), methyl heptadecanoate as an internal standard, split ratio of 1:50 and 25 min analysis time. Samples were prepared by dissolution of 50 mg of the washed and dried sample into 1 mL of internal standard solution (10 mg mL^{-1}, in heptane) and 1 μL was applied to the equipment. The ester mass yield (wt.%) was calculated in accordance with Equation 5:

$$\text{Ester yield (wt.\%)} = \frac{(\sum A) - A_{SI}}{A_{SI}} \times \frac{C_{SI} \times V_{SI}}{m} \times 100 \quad (5)$$

where C_{SI} corresponds to concentration of the internal standard (10 mg mL^{-1}), A_{SI} corresponds to peak area of the internal standard (C17), ΣA is the total peak area of fatty acid ethyl esters C14:0 to C24:0, V_{SI} and m corresponds to volume of the internal standard solution (1 mL) and mass of sample (50 mg), respectively.

Free fatty acids were analyzed in accordance with methodology adapted from the Agilent Technologies Catalog (2011). For this purpose, a 7890A Agilent chromatograph (Santa Clara, CA, USA) with Rtx-Wax capillary column (30 m × 0.25 mm × 0.25 μm, Restek Corporation, Bellefonte, PA, USA) and FID detector (settled at 250 °C) was used. The oven temperature was set at 120 °C for 1 min; 120-250 °C to 10 °C min^{-1}; 250 °C for 5 min. Helium was used as carrier gas (42 cm s-1, 24 psi at 120 °C, 1.8 mL min^{-1}). The samples were prepared by dissolving 16 mg of the washed and dried sample in 10 mL of dichloromethane (1.6 mg mL^{-1}), and the standards (palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid) were prepared in five different concentrations (0.25, 0.5, 1.0, 1.5 and 2.0 g L^{-1}) to adjust the calibration curve.

10.2.2.9.2 ATR-FTIR

Fourier transform infrared spectrophotometer (ATR-FTIR) (Vertex 70 model, Bruker, Germany) was used. This equipment features a diamond crystal single bounce accessory. Sample spectra were determined after 32 scans between 4000 and 400 cm^{-1} with a spectral resolution of 4 cm^{-1}.

10.3 Results and Discussion

10.3.1 Physicochemical characterization of the oil

The physicochemical analyses of the DSO used for the hydroesterification reaction are shown in Table 10.2. The saponification index is within the range (189–195 mg KOH g^{-1} oil) reported for soybean oil [62], while the acidity index value is higher than that observed for this oil after refining [7]. The value for the degree of unsaturation measured by the iodine index is in agreement with reports from Verastegui et al. [63].

Table 10.2. Physicochemical analysis for DSO.

Parameter	Values
Acid index (mg KOH/g) (25 °C)	1.43 ± 0.15
Saponification index (mg KOH/g) (25 °C)	196.51 ± 1.90
Iodine index (g I_2/100 g) (25 °C)	135.42 ± 4.52
Kinematic viscosity (mPa.s) (40 °C)	33.76 ± 0.51
Water content (%) (130 °C)	0.55 ± 0.05

10.3.2 Optimized enzymatic hydrolysis to produce FFA

10.3.2.1 Screening of the lipase used in hydrolysis

Figure 10.1 shows the screening of the lipases studied in DSO hydrolysis. PFL demonstrated a performance superior to the other lipases, reaching a conversion 18 times higher than the CALB. This result may be related to the specificity of lipases, since PFL is a non-specific enzyme [64,65]. Besides that, can be explained based on the fact that some lipases have high activity for short and medium chain fatty acids and decreasing activity for long-chain fatty acids [66]. Thus, it is adequate to propose that PFL is the best biocatalyst to be used with DSO hydrolysis considering that this oil is composed mostly of long-chain fatty acids (linoleic and oleic acids).

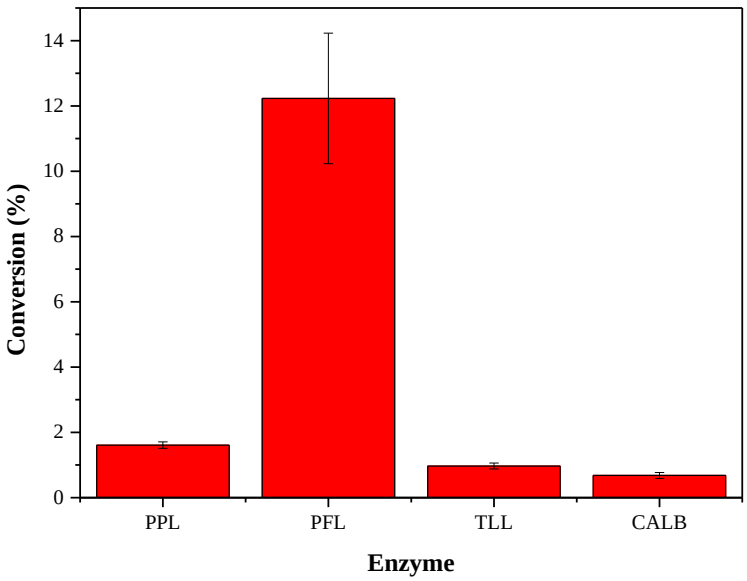

Figure 10.1. Biocatalyst screening for the hydrolysis of DSO. Assay conditions: enzyme load of 25 U/g oil, oil/water mass ratio of 1:4, temperature of 35 °C, agitation of 250 rpm for 5 min.

10.3.2.2 Effect of water proportion on DSO hydrolysis

The effect of different mass ratios of oil/water in the hydrolysis of DSO was also evaluated. Figure 10.2 shows that the highest conversion to FFA (around 65%) was achieved with a mass ratio of 1:4. For the other oil/water mass ratios there was a small reduction in the reaction conversion. Thus, the oil/water mass ratio of 1:0.5 was chosen for further assays. This ratio was selected because it allows the use of a working volume of 66.7% with organic medium and facilitates the process of free enzyme reuse, when the amount of glycerol accumulates in the system.

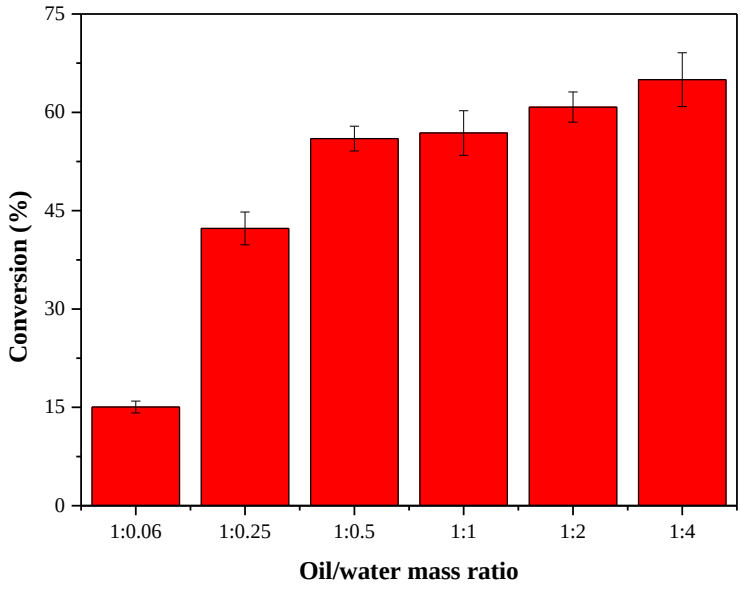

Figure 10.2. Effect of the oil/water mass ratio in hydrolysis of DSO catalyzed by PFL. Assay conditions: enzyme load of 50 U/g oil, temperature of 35 °C, agitation of 250 rpm for 24 h.

10.3.2.3 Enzyme loading

The effect of the enzyme load on the hydrolysis of the DSO as a function of the reaction time showed that there is an increase in the conversion of FFA as the enzyme load was increased from 50 U/g oil (55.47% conversion) to 250 U/g oil (61.24% conversion). However, this small increase does not justify the use of high enzyme loads in this reaction. An appropriate enzyme load is a critical factor in the economic viability of the process. In this sense, an enzyme load of 50 U/g oil was selected to investigate the kinetic profile of the hydrolysis of DSO catalyzed by PFL.

10.3.2.4 Determination of the optimal operating time

Figure 10.3 shows the kinetic profile of the hydrolysis of DSO catalyzed by PFL. A maximum conversion of 97% is observed with 23 h of reaction. After that time, no significant increase in FFA conversion was observed. These results are promising since the reaction was carried out in a system without emulsifier and without buffer, being composed only of DSO, water and enzyme, eliminating the need for FFA purification steps and reducing process costs. The composition of the FFAs were 10.30 ± 0.17 wt.% of palmitic acid, 6.29 ± 0.03 wt.% of stearic acid, 22.80 ± 0.25 wt.% of oleic acid, 45.25 ± 0.27 wt.% of linoleic acid and 8.85 ± 0.04 wt.% of linolenic acid , which accords with the composition of soybean oil [37]. The time of 24 h was selected to evaluate the reuse of the enzyme, together with the heavy phase (water, glycerol and enzyme).

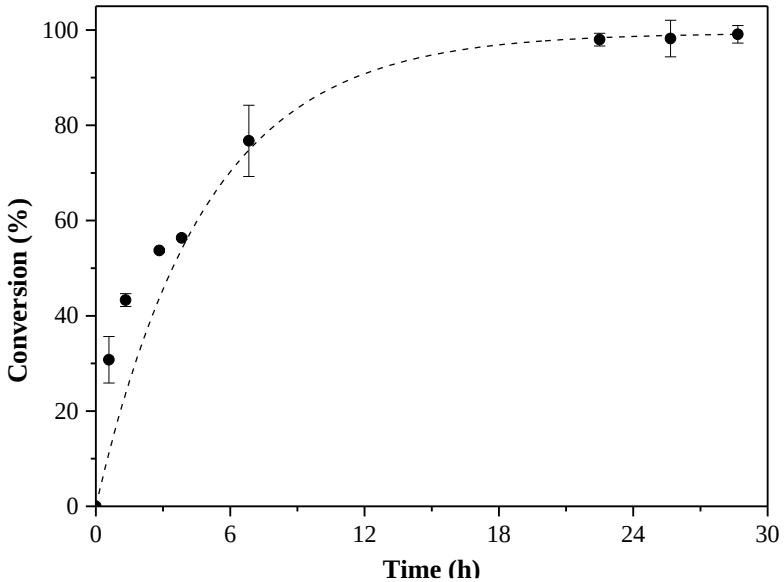

Figure 10.3. Time course of hydrolysis of DSO catalyzed by PFL. Assay conditions: enzyme load of 50 U/g DSO, oil/water mass ratio of 1:0.5, temperature of 35 °C, 900 rpm stirring in a batch reactor.

10.3.2.4.1 Reuse of free PFL

Figure 10.4 shows the reuse of PFL in the hydrolysis of DSO. It is observed that it was possible to reuse the heavy phase of the hydrolysis reaction for at least 3 sequential batches, keeping the conversion above 61%, when there was no use of hexane to recover the heavy phase.

The reduction in the performance of the hydrolysis reaction may have been caused by several factors, including: (1) loss of enzyme in the separation stages of the heavy phase, since

reaction intermediates (mono- and diglycerides) emulsify and hinder the efficient separation of the phases (it was observed that after decantation, the heavy phase visually showed yellowish opacity, being a qualitative indicative of emulsification); (2) reduced catalytic activity due to adsorption on the surface of the enzyme products and reaction intermediates (free fatty acids, mono- and diglycerides) [67]. In order to verify these hypotheses, at the end of the fifth batch, sodium chloride was added to the reaction medium, in an amount close to its maximum solubility in water at room temperature (36 g NaCl/100 g water). The heavy phase was then reused in a new batch of DSO hydrolysis, achieving a conversion of 38% after 24 h of reaction. This demonstrates that the conversion in the fifth batch (12% conversion) is partly due to the reduction in catalytic activity due to the adsorption of free fatty acids and/or non-converted acylglycerols to the enzyme surface. This theory was validated when the light phase was recovered with hexane at the end of each recycle (Figure 10.4). In this case, there is a reduction in the conversion of 20% in the second cycle, however, there is a stabilization, reaching 65% of conversion in the fifth cycle. The use of solvent proved to be a viable alternative for recovering the heavy phase in the hydrolysis step, considering the possibility of reuse the heavy phase and the hexane in subsequent recycles. In fact, after each batch, hexane was recovered by rota-evaporation and reused in the next cycle.

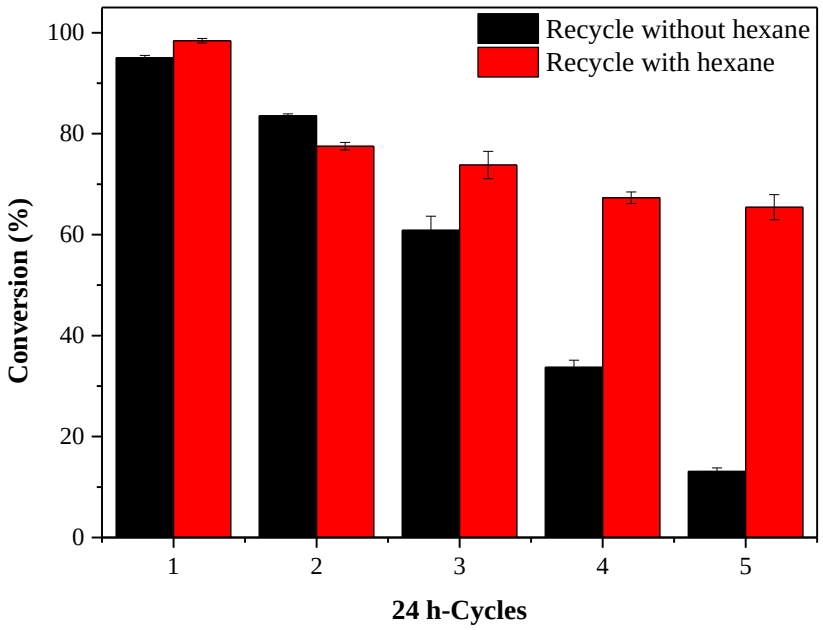

Figure 10.4. Reuse assay (24 h-cycles) of the PFL in the hydrolysis of DSO. Assay conditions: enzyme load of 50 U/g DSO, oil/water mass ratio of 1:0.5, temperature of 35 °C, 900 rpm stirring in a reactor with mechanical agitation.

The strategy of reusing the heavy phase can maximize the economic viability of the free fatty acid production process. In addition, it allows the concentration of glycerol with high purity be used in other process of food, cosmetic and pharmaceutical purposes.

10.3.3 Enzymatic esterification to produce octyl esters

EV, CALB and PPL were immobilized on Purolite for further application in the synthesis of octyl esters in a solvent-free medium. Figure A10.1 shows immobilization courses for the three enzymes. Immobilization was very rapid, with more than 80% of protein adsorption after 1 h. The recovered activity and immobilization yield of the biocatalysts are presented in Table A10.1. EV and CALB showed high immobilization yield, however, low recovered activity. On the other hand, the opposite behavior was evidenced for the PPL. The three biocatalysts and the Lipozym 435 (a commercial immobilized CALB) were used for the subsequent esterification step.

As shown in Figure 10.5, the non-commercial biocatalysts, with exception of PPL-Purolite, showed behavior equal to or greater than Lipozym 435. EV-Purolite reached ester yield around 86% after 24 h of reaction. These results demonstrate that immobilization on Purolite Lifetech EC8806F produced a competitive biocatalyst to the commercial immobilized lipases.

The EV-Purolite was selected to evaluate the influence of the time on the octyl esters synthesis. The use of EV in the form of magnetic cross-linked enzyme aggregate for the production of isoamyl esters by transesterification of waste cooking oil has already been demonstrated [7]. However, there is no evidence in the literature of the application of this enzyme to the production of octyl esters by hydroesterification of DSO.

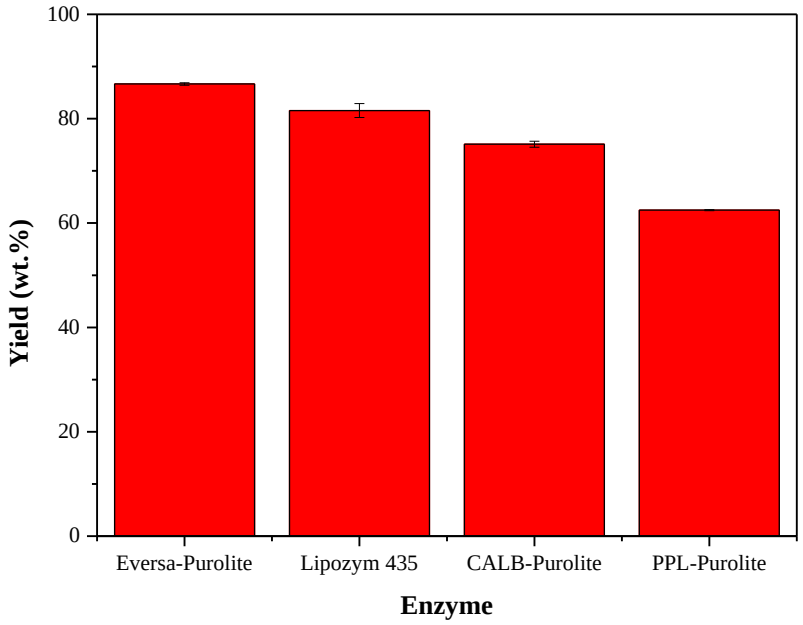

Figure 10.5. Biocatalyst screening for esterification of the FFA-rich fraction from the DSO hydrolysis. Assay conditions: enzyme load of 1% (w/v), FFA/octanol molar ratio of 1:2.5, temperature of 37 °C, agitation of 250 rpm for 24 h.

Figure 10.6 shows that, using EV-Purolite, the higher enzyme load, the higher the esterification initial rate, however there is no effect on the synthesis kinetics of the octyl esters. Using 1% (w/v) of enzyme load, 94.11 ± 0.5 wt% of the octyl esters yield was achieved after 3 h of reaction. After that time, no significant increase was observed in the esters yield. As this is a kinetically controlled reaction and the octyl esters is a substrate of the enzyme, some hydrolysis of these esters may be expected if the thermodynamics of the process allows it. Thus, a time of 3 h was used to analyze the operational stability of EV-Purolite.

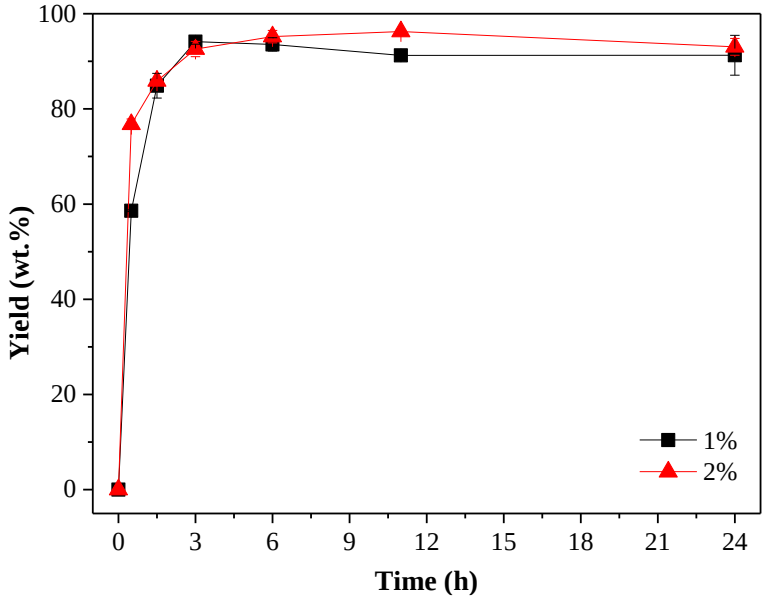

Figure 10.6. Time course of esterification of FFA with octyl alcohol catalyzed by EV-Purolite. Assay conditions: FFA/octyl molar ratio of 1:2.5, temperature of 37 °C, 1500 rpm stirring in a vortex-type batch reactor. Solid squares: enzyme load of 1% (w/v) and solid triangles: enzyme load of 2% (w/v).

The operational stability (reuse assays) of the EV-Purolite is shown in Figure 10.7. The yield of octyl esters was retained after five 3 h-batches. This result shows that immobilized biocatalyst had high operational stability, indicating strong hydrophobic interactions and stable complex formation between enzyme and support. These characteristics are fundamental aspects for the application of immobilized enzymes in industrial processes. Another advantage of using EV immobilized on Purolite Lifetech EC8806F is the possibility of recyclability of the support after enzyme inactivation [40].

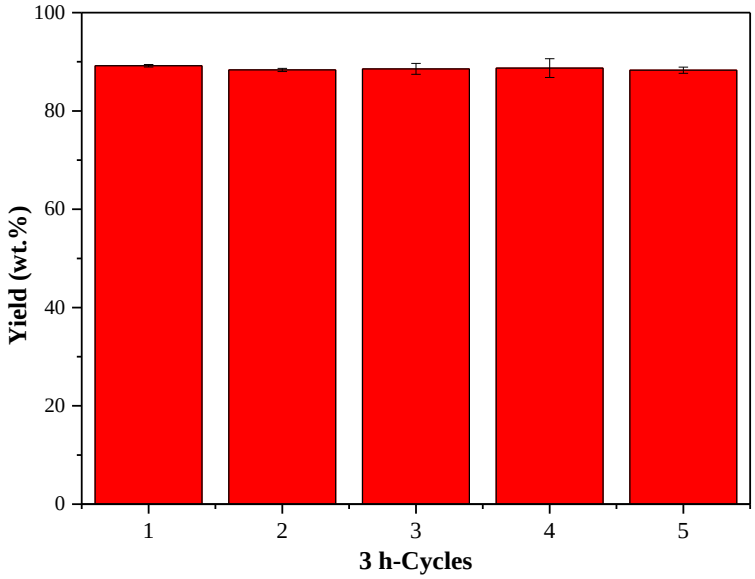

Figure 10.7. Reuse assay (3 h-cycles) of EV-Purolite (enzyme load of 1%, w/v) in the esterification of FFA with octyl alcohol (molar ratio of 1:2.5) at 37 °C in a batch vortex flow reactor stirred initially at 1500 rpm and gradually increased up to 2000 rpm.

The synthesis of octyl esters was confirmed by ATR-FTIR spectra. Figure 10.8 shows the ATR-FTIR spectra of octyl alcohol, FFA and octyl esters. The octyl alcohol presents an intense band at 3335 cm^{-1} which is attributed to the O–H stretching absorption, while the band at 1038 cm^{-1} is attributed to the C–O single bond stretching vibration of a primary alcohol. It is observed that the intense band at 1709 cm^{-1} corresponding to the carbonyl group of the FFA was changed to 1738 cm^{-1} after the esterification reaction. In addition, it is possible to verify the band at 1173 cm^{-1} that correspond to the vibrational elongation of the –C (=O) O– group, thus confirming the synthesis of fatty acid octyl esters. This ATR-FTIR spectrum profile agrees with previous findings of biolubricants synthesized by esterifying FFA from waste cooking oil and octyl alcohol catalyzed by Novozyme 435 [58].

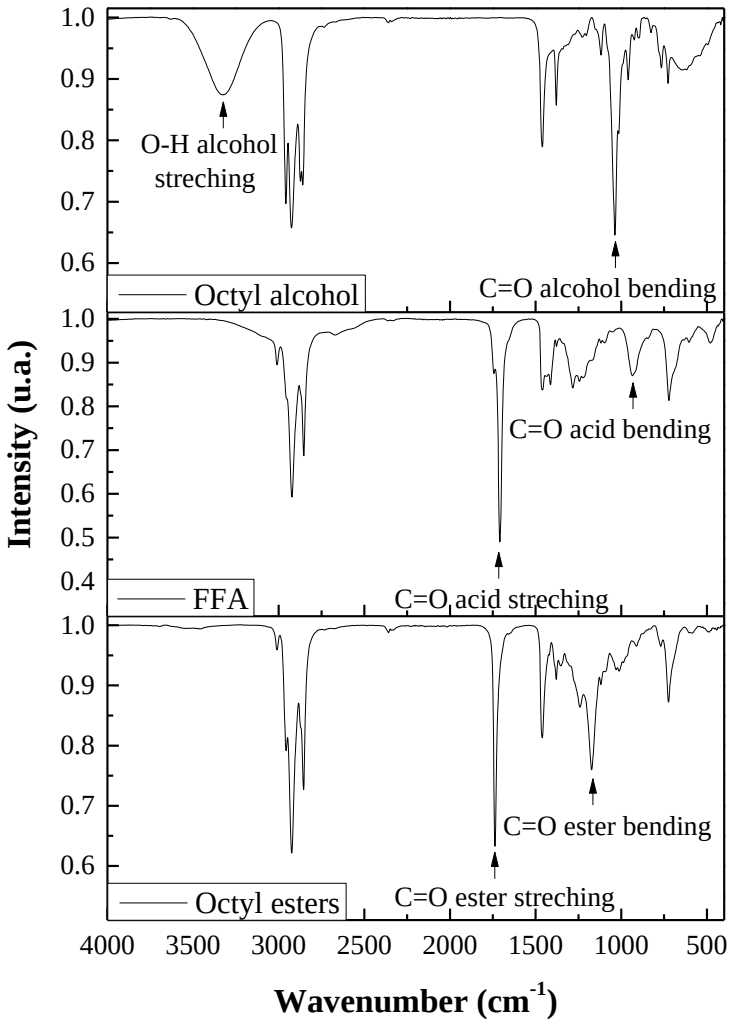

Figure 10.8. ATR-FTIR spectra of octyl alcohol, FFA and octyl esters. Before carrying out the analysis, the esters were dried at 135 ºC for 16 h.

10.4 Conclusion

This paper shows the feasibility of the enzymatic approach used for the enzymatic synthesis of octyl alcohol-bases biolubricants carried out in two steps. The use of free PFL in DSO hydrolysis allowed a high FFA yield, performed in an emulsifier-free system with no buffer, being only composed of DSO, water and enzyme, thus eliminating the need of FFA purification steps and reducing the process costs. Furthermore, the possibility of using the heavy phase of the hydrolysis reaction in sequential batches of DSO hydrolysis was demonstrated. In the esterification step, the EV immobilized on Purolite Lifetech EC8806F successfully provided the synthesis of octyl esters (around 94 wt.% of ester yield). The immobilized lipase could be reused in five 3 h-batches of esterification, maintaining 100% of the octyl esters yield of the

first batch. The nature of the product was confirmed by spectroscopy (ATR-FTIR) and gas chromatography (GC) analyses.

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APPENDIX

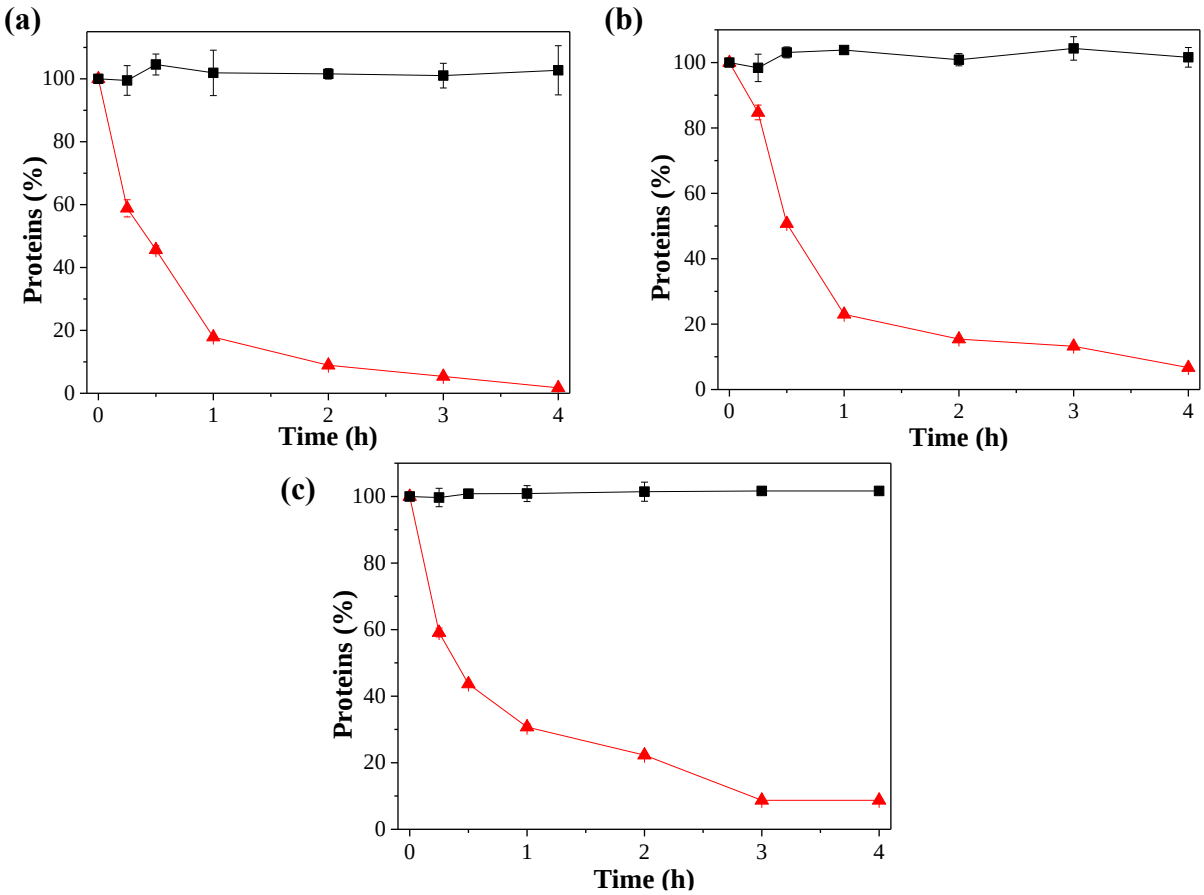

Figure A10.1. Immobilization courses of (a) EV, (b) CALB and (c) PPL on Purolite Lifetech EC8806F (20 mg/g). Immobilization conditions: 5 mM of sodium phosphate buffer at pH 7.0, temperature of 25 °C (for EV and CALB) and 5 °C (for PPL), and gentle shaking. Solid squares: reference and solid triangles: suspension. Other specifications are described in Methods.

Table A10.1. Evaluation of the immobilization parameters of EV, CALB and PPL on Purolite Lifetech EC8806F (20 mg/g).

Biocatalyst	RA (%)	IY (%)
CALB-Purolite	11.58 ± 0.46	98.87 ± 0.10
EV-Purolite	24.88 ± 1.32	94.00 ± 0.12
PPL-Purolite	49.54 ± 2.17	80.84 ± 0.18

Immobilization conditions: 5 mM of sodium phosphate buffer at pH 7.0, temperature of 25 °C (for EV and CALB) and 5 °C (for PPL), and gentle shaking. For all enzymes the protein offered to the immobilization was 40 mg/g support. Other specifications are described in Methods.

CHAPTER 11
CONCLUSIONS, SUGGESTIONS FOR FUTURE WORKS, AND ACADEMIC PRODUCTION

In this thesis, it was demonstrated as a library of biocatalysts can be developed combining enzyme immobilization strategies with mineralization of the immobilized enzymes. Here the general conclusions obtained in this thesis will be summarized.

11.1 Conclusions

The concepts of systematic mapping (SM) showed the direction of research related to the immobilization of lipases on hydrophobic supports, their bottlenecks and solutions to problems. With that, the proposed of mineralization of immobilized enzymes was suggested.

The modification of immobilized enzymes with metallic phosphate enable to have some of the advantages achieved using the nanoflower production method to immobilize lipase. This strategy can be of general applicability to other immobilized enzymes, with the advantage of the possibility of selecting the mechanical properties of the final biocatalyst. Commercial preparations of immobilized lipases modified with metal phosphate alters enzyme specificity.

The same immobilization protocol (interfacial activation) using supports with different degrees of hydrophobicity can generate different enzymatic conformations, and thus mineralization can have different effects on enzymatic characteristics. It has also been demonstrated on the power of immobilization to tune enzyme functional features, including examples where the only difference is the final physical interactions between enzyme and support (i.e., octyl-vinyl sulfone biocatalysts blocked with different reagents). As well as, when the only difference between the different biocatalysts is the groups located surface of the support, and therefore, the enzyme-support interactions. Therefore, the use a wide battery of metals and immobilization protocols can permit to “create biocatalysts” adequate for many processes, even if the original enzyme does not look very adequate for the specific process. Furthermore, the mineralization of chemically or physically modified immobilized enzymes is a potent tool to improve enzyme features, but the effects cannot be predicted at this stage and must be empirically analyzed. The same mineralization could present different effects on the enzyme activity, specificity or stability, depending on the previous modification performed on the enzyme, showing that these previous enzyme modifications alter the effects of the mineralization on enzyme features. In this way, the combination of chemical or physical

modifications of enzymes before their mineralization increases the range of modification of features that the immobilized enzyme can experienced, enabling to enlarge the biocatalyst library.

Finally, Eversa[@] Transform immobilized on Purolite[@] C18 was successfully applied in the esterification of purified fatty acids of the hydrolysis of degummed soybean oil for the synthesis of octyl esters. Furthermore, the viability of the enzymatic approach for the production of methyl esters using TLL immobilized on Purolite[@] C18 aminated and activated with vinyl sulfone was demonstrated.

11.2 Conclusiones

Los conceptos de mapeo sistemático (MS) mostraron el direccionamiento de las investigaciones relacionadas a la inmovilización de lipasas en soportes hidrofóbicos, sus lagunas y soluciones de problemas. Por consiguiente, se sugirió la propuesta de mineralización de enzimas inmovilizadas.

La modificación de enzimas inmovilizadas con fosfato metálico permite obtener algunas de las ventajas alcanzadas por el método de producción de nanoflowers para inmovilizar lipasa. Esta estrategia puede ser de aplicabilidad general para otras enzimas inmovilizadas, con la ventaja de la posibilidad de seleccionar las propiedades mecánicas del biocatalizador final. Los preparados comerciales de lipasas inmovilizadas modificadas con fosfato metálico alteran la especificidad de la enzima.

El mismo protocolo de inmovilización (activación interfacial) utilizando soportes con diferentes grados de hidrofobicidad puede generar diferentes conformaciones enzimáticas y, por lo tanto, la mineralización puede tener diferentes efectos en las características enzimáticas. También se ha demostrado el poder de la inmovilización para ajustar las características funcionales de la enzima, incluyendo ejemplos en los cuales la única diferencia son las interacciones físicas finales entre la enzima y el soporte (es decir, biocatalizadores de octil-vinil sulfona bloqueados con diferentes reactivos). Así como, cuando la única diferencia entre los diferentes biocatalizadores son los grupos ubicados en la superficie del soporte y por lo tanto las interacciones enzima-soporte. Por lo tanto, el uso de una amplia batería de metales y protocolos de inmovilización pueden permitir "crear biocatalizadores" adecuados para muchos procesos, aunque la enzima original no parezca muy adecuada para el proceso específico. Además, la mineralización de enzimas inmovilizadas químicamente o físicamente modificadas es una herramienta potente para mejorar las características de la enzima, pero los efectos no

pueden predecirse en esta fase y deben analizarse empíricamente. Una misma mineralización puede presentar diferentes efectos sobre la actividad, especificidad o estabilidad de la enzima, dependiendo de la modificación anterior realizada en la enzima, mostrando que esas modificaciones enzimáticas anteriores alteran los efectos de la mineralización en las características de la enzima. De esta forma, la combinación de modificaciones químicas o físicas de enzimas antes de su mineralización aumenta la gama de modificaciones de características que la enzima inmovilizada puede experimentar, permitiendo ampliar la biblioteca de biocatalizadores.

Por último, Eversa[@] Transform inmovilizado en Purolite[@] C18 fue aplicada con éxito en la esterificación de ácidos grasos purificados de la hidrólisis de aceite de soja desgomado para la síntesis de octil ésteres. Además, se ha demostrado la viabilidad del enfoque enzimático para la producción de ésteres metílicos utilizando TLL inmovilizado en Purolite[@] C18 aminado y activado con grupos vinilo sulfónico.

11.3 Suggestions for future works

Due to the results shown in this thesis and aware that there are still many gaps to be studied, below are some suggestions for future work:

- Mineralization of other classes of immobilized enzymes with different metallic phosphates;
- Preparation of enzymatic aggregates in the presence of different metallic phosphates;
- Application in different organic reactions of biocatalysts obtained by different methods of immobilization and mineralization with different metallic phosphate;
- Application in different organic reactions of biocatalysts obtained by different physical and/or chemical modifications and mineralization with different metallic phosphate.

11.4 Academic production

The results of this thesis were published in indexed journals, as listed below:

- **J.R. Guimarães**, D. Carballares, J. Rocha-Martin, A.R. Alcántara, P.W. Tardioli, R. Fernandez-Lafuente. Heterofunctional Methacrylate beads bearing octadecyl

and vinyl sulfone groups: Tricks to obtain an interfacially activated lipase from *Thermomyces lanuginosus* and covalently attached to the support. Catalysts, 13 (2023) 108, 2023. https://doi.org/10.3390/catal13010108. IF: 4.501.

- **J.R. Guimarães**, R. Fernandez-Lafuente, P.W. Tardioli. Ethanolysis of soybean oil catalyzed by magnetic CLEA of porcine pancreas lipase to produce ecodiesel. Efficient separation of ethyl esters and monoglycerides. Renewable Energy, 198 (2022) 455-462. https://doi.org/10.1016/j.renene.2022.08.033. IF: 8.634.
- **J.R. Guimarães**, D. Carballares, J. Rocha-Martin, P.W. Tardioli, R. Fernandez-Lafuente. Stabilization of immobilized lipases by treatment with metallic phosphate salts. International Journal of Biological Macromolecules, 213 (2022) 43-54. https://doi.org/10.1016/j.ijbiomac.2022.05.167. IF: 8.025.
- **J.R. Guimarães**, D. Carballares, J. Rocha-Martin, P.W. Tardioli, R. Fernandez-Lafuente. Tuning immobilized enzyme features by combining solid-phase physicochemical modification and mineralization. International Journal of Molecular Sciences, 23 (2022) 12808. https://doi.org/10.3390/ijms232112808. IF: 6.208.
- **J.R. Guimarães**, D. Carballares, J. Rocha-Martin, P.W. Tardioli, R. Fernandez-Lafuente. The immobilization protocol greatly alters the effects of metal phosphate modification on the activity/stability of immobilized lipases. International Journal of Biological Macromolecules, 222 (2022) 2452-2466. https://doi.org/10.1016/j.ijbiomac.2022.10.030. IF: 8.025.
- **J.R. Guimarães**, D. Carballares, P.W. Tardioli, J. Rocha-Martin, R. Fernandez-Lafuente. Tuning immobilized commercial lipase preparations features by simple treatment with metallic phosphate salts. Molecules, 27 (2022) 4486. https://doi.org/10.3390/molecules27144486. IF: 4.925.
- **J.R. Guimarães**, D. Carballares, J. Rocha-Martin, P.W. Tardioli, R. Fernandez-Lafuente. Mineralization of lipase from *Thermomyces lanuginosus* immobilized on methacrylate beads bearing octadecyl groups to improve enzyme features. Catalysts, 12 (2022) 1552. https://doi.org/10.3390/catal12121552. IF: 4.501.
- **J.R. Guimarães**, L.P. Miranda, R. Fernandez-Lafuente, P.W. Tardioli. Immobilization of Eversa® Transform via CLEA technology converts it in a suitable biocatalyst for biolubricant production using waste cooking oil. Molecules, 26 (2021) 193. https://doi.org/10.3390/molecules26010193. IF: 4.927.

During the doctorate, partnerships were established, and some works were published in journals indexed as co-author, as listed below:

- M.C.P. Gonçalves, J.P. Romanelli, A.B.M Cansian, E.F.Q. Pucci, **J.R. Guimarães**, P.W. Tardioli, B.A. Saville. A review on the production and recovery of sugars from lignocellulosics for use in the synthesis of bioproducts. Industrial Crops and Products, 186 (2022) 115213. https://doi.org/10.1016/j.indcrop.2022.115213. IF: 6.449.
- T.N. Da Rocha, D. Carballares, **J.R. Guimarães**, J. Rocha-Martin, P.W. Tardioli, L.R.B. Gonçalves, R. Fernandez-Lafuente. Determination of immobilized lipase stability depends on the substrate and activity determination condition: Stress inactivations and optimal temperature as biocatalysts stability indicators. Sustainable Chemistry and Pharmacy, 29 (2022) 100823. https://doi.org/10.1016/j.scp.2022.100823. IF: 5.464.
- M.C.P. Gonçalves, J.P. Romanelli, E.F.Q. Pucci, **J.R. Guimarães**, A.C. Vieira, B.P. Azevedo, P.W. Tardioli. Reviewing research on the synthesis of CALB-catalyzed sugar esters incorporating systematic mapping principles. Critical Reviews in Biotechnology, 1 (2021) 1-23. https://doi.org/10.1080/07388551.2021.1888071. IF: 9.062.
- A.C. Vieira, A.B.M. Cansian, **J.R. Guimarães**, A.M.S. Vieira, R. Fernandez-Lafuente, P.W. Tardioli. Performance of liquid Eversa on fatty acid ethyl esters production by simultaneous esterification/transesterification of low-to-high acidity feedstocks. Catalysts, 11 (2021) 1486. https://doi.org/10.3390/catal11121486. IF: 4.501.
- L.P. Miranda, **J.R. Guimarães,** R.C. Giorgano, R. Fernandez-Lafuente, P.W. Tardioli. Composites of crosslinked aggregates of Eversa® Transform and magnetic nanoparticles. Performance in the ethanolysis of soybean oil. Catalysts, 10 (2020) 817. https://doi.org/10.3390/catal10080817. IF: 4.501.