



UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

**Chemical Modification of Proteins, Applications in
Immobilization, Catalytic Performance and Fluorescent
Labeling**

**Modificación Química de Proteínas, Aplicaciones
en Inmovilización, Actividad Catalítica y
Marcaje Fluorescente**

D^a. Samanta Hernández García

2017

UNIVERSIDAD DE
MURCIA



Universidad de Murcia

Facultad de Biología

“Modificación química de proteínas, aplicaciones en inmovilización, actividad catalítica y marcaje fluorescente.”

“Chemical modification of proteins, applications in immobilization, catalytic performance and fluorescent labeling.”

Memoria presentada para optar al título de doctor por la Universidad de Murcia.

Samanta Hernández García

marzo de 2017



UNIVERSIDAD DE
MURCIA

D. Francisco García Carmona, Catedrático de Universidad del Área de Bioquímica en el Departamento de Bioquímica y Biología Molecular A, AUTORIZA:

La presentación de la Tesis Doctoral titulada "Modificación química de proteínas, aplicaciones en inmovilización, actividad catalítica y marcaje fluorescente", realizada por D^a. Samanta Hernández García, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

marzo de 2017



UNIVERSIDAD DE
MURCIA

D^ª. María Inmaculada García García, Doctora de Universidad del Área de Bioquímica colaboradora en el Departamento de Bioquímica y Biología Molecular A, AUTORIZA:

La presentación de la Tesis Doctoral titulada “Modificación química de proteínas, aplicaciones en inmovilización, actividad catalítica y marcaje fluorescente“, realizada por D^ª. Samanta Hernández García, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

marzo de 2017

La siguiente tesis doctoral ha sido parcialmente subvencionada por los siguientes proyectos:

MINECO-FEDER (AGL2011-25023)

Programa de Ayuda a Grupos de Excelencia de la Región de Murcia, Fundación Séneca (04541/GERM/06, Plan Regional de Ciencia y Tecnología 2007-2010).

“Ministerio de Economía y Competitividad” (MEC, FEDER, España) Proyecto AGL 2014-57431

“Programa de ayudas a Grupos de Excelencia de la Región de Murcia, de la Fundación Séneca, Agencia de Ciencia y Tecnología de la Región de Murcia” nº 19893/GERM/2015.

El contenido de esta tesis ha dado como resultado los siguientes manuscritos científicos:

PUBLICADOS

Patente **Funcionalización de partículas magnéticas mediante sustitución nucleofílica de haluros orgánicos** ES2540026B1/WO2015092106A1

Fecha de Publicación 7/7/2015

Inventores: Francisco García Carmona, Samanta Hernández García, María Inmaculada García García

CONGRESO

Hernández García S., Zapata Pérez R., Martínez Moñino A.B., García Carmona F. "Immobilization and characterization of trametes versicolor lacasse in porous silica particles". FEBS JOURNAL, VOL. 280, 108--108, (2013)

ENVIADOS

1-"Improving the production, activity and stability of CLEAs with diepoxides"

Biotechnology Progress, BTPR-16-0387

Autores: Samanta Hernández García, María Inmaculada García García, Francisco García Carmona

2-"Cinnabarinic acid an active compound: Characterization and Synthesis by immobilized Trametes versicolor laccase."

Journal of Biotechnology, JBIOTEC-D-16-01714

Autores: Samanta Hernández García, María Inmaculada García García, Francisco García Carmona

3-"Epoxide-based immobilization of Laccase inside mesocellular foam as green catalyst for dyes synthesis."

Microporous & Mesoporous Materials

Autores: Samanta Hernández García, Maria Inmaculada García García, Francisco García Carmona

4-"Preparation of magnetic epoxy cross-linked enzyme aggregates of Aldolase"

Biocatalysis and Biotransformation, GBAB-17-1009

Autores: Samanta Hernández García, Maria Inmaculada García García, Francisco García Carmona

5-An improved method to measure Lipase activity in aqueous media."

Analytical Biochemistry, ABIO-17-54

Autores: Samanta Hernández García, Maria Inmaculada García García, Francisco García Carmona

PROPUESTA DE PATENTE

“Síntesis de derivados fluorescentes con aplicaciones en marcaje de proteínas, células y ácidos nucleicos.”

Inventores: Francisco García Carmona, Samanta Hernández García, María

Inmaculada García García

“Esta tesis doctoral está sometida a procesos de protección o transferencia de tecnología o de conocimiento, por lo que los siguientes contenidos están inhibidos en la publicación en los repositorios institucionales.

Autorizado por la Comisión General de Doctorado de la Universidad de Murcia
con fecha de Marzo 2017”

Relación de Contenidos Excluidos

Chapter 11: Protein labeling and gel staining using synthesized low cost fluorophores.

Chapter 12: Synthesized hydrophobic C18 Fluorophores for protein labeling, cell signaling and protein quantification.

Chapter 13: HIS-tag proteins affinity labeling with synthesized fluorescent Nickel coordinated NTA.

Agradecimientos

En primer lugar mi más sincero agradecimiento al Catedrático Dr. D. Francisco García Carmona por la gran oportunidad de realizar esta tesis doctoral, bajo su dirección y tutela en el grupo de investigación Bioquímica y Biotecnología Enzimática. Paco te estoy muy agradecida por guiarme y enseñarme a lo largo de toda tesis doctoral.

A mi codirectora Dra. D^a. María Inmaculada García García tengo mil cosas que agradecerle, ya que ha contribuido a esta tesis no solo como codirectora, sino también como “profesora” y sobretodo como compañera y amiga, Muchas gracias María!

A todos y cada uno de los miembros del grupo de investigación Bioquímica y Biotecnología Enzimática quiero agradecerles el haberme acogido en departamento. Al Dr. D. José Manuel López Nicolás, al Dr. D. Fernando Gandía Herrero, a las profesoras Dra. D^a. Juana Cabanes, Dra. D^a. Mercedes Jimenez, y Dra. D^a. Josefa Escribano, a la Dra. D^a Manuela Pérez y al Dr. D. Álvaro Sánchez.

A los doctorandos, a Rubén y Anabel con los que empecé esta aventura hace casi cinco años. Rubén mi compi de laboratorio, el L7 ha sido nuestro bastión Heavy. A Antonio por ser Antonio (no cambies nunca!). A Adrián por esas duras mañanas delante del espectrofotómetro con el dichoso trans-metilestilbena (que no se oxidaba). A Karol por ir todos los días con una sonrisa. A Berenice

por su alegría y pasión por la investigación. A Sandra por ser encantadora y ayudarme con las imágenes del microscopio. Muchas gracias a todos por todos estos años.

A Alejandro, Aurora, Ana, Yolanda y M. Dolores sois los mejores, empezamos juntos en el Aulario general y hemos compartido muchas cosas, risas, alguna que otra lagrima y muchas más risas. Estéis lejos o cerca, nos veamos más o menos siempre os llevo conmigo.

A mi familia. A mis Padres, esta tesis es en gran parte vuestra, sin vosotros JAMÁS hubiera llegado hasta aquí. Mama, Papa Muchísimas Gracias! Os quiero!

Doy las gracias también la sección de Microscopia de la Universidad de Murcia, por las imágenes del TEM y del microscopio confocal.

A todos Vosotros

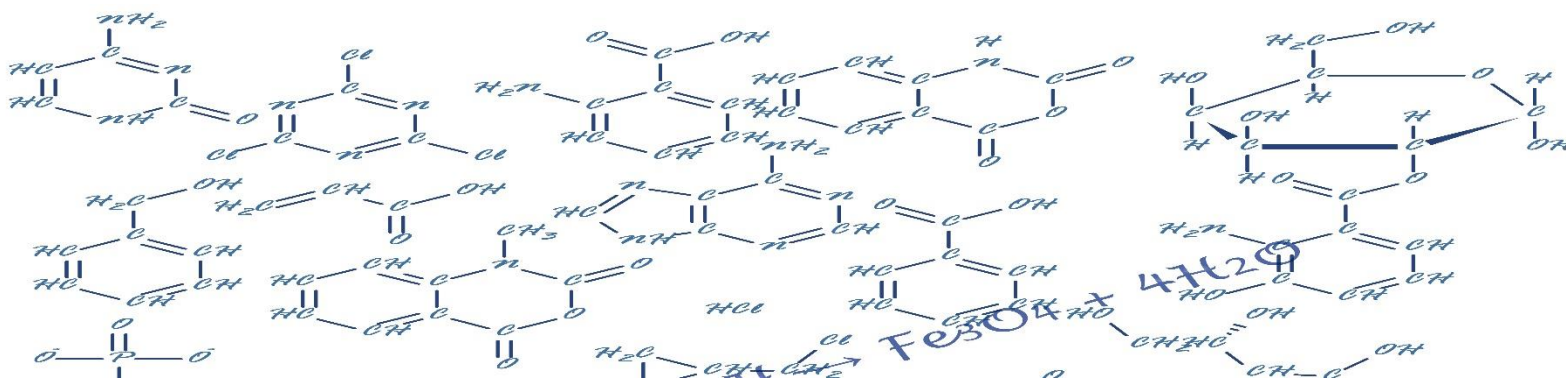
GRACIAS!

A mis Padres



When life give you lemons, make grape juice. Then sit back and watch the world wonder how the hell you did it.

Index



Index

	PAGE
Chapter 1: Introduction: Chemical Modification of Proteins	1
Overview	
1 Chemical modification of proteins	3
2 Enzyme immobilization overview	5
2.1 Immobilization by Cross-linking (carrier free immobilization)	7
2.2 Carrier bond immobilization	9
2.2.1 Binding techniques	9
2.2.2 Materials for supports preparation	10
2.3 Immobilization by entrapment	12
3 Protein fluorescent labeling	13
3.1 Fluorophores for labeling	14
Chapter 2: Objectives	17
Chapter 3: Materials and Methods.	21
1 Materials	23
1.1 Chemical compounds	23
1.2 Proteins	23
2 Methods	24
2.1 Enzymes general activity test	24
2.1.1 Aldolase activity measurements	24
2.1.2 Laccase activity test	24
2.1.3 Lipase activity test	25
2.2 Protein quantification	26
2.2.1 Bradford method	26

2.2.2 Bicinchoninic method	27
2.3 Protein Electrophoresis	27
2.3.1 Acrylamide SDS-PAGE gels	27
2.3.2 Sample Denaturalization and Loading	28
2.3.3 Colloidal Coomassie Brilliant Blue Gel Stain	28
2.4 Siliceous supports preparation	29
2.4.1 Fumed silica nanoparticles (FsNp)	29
2.4.2 Spherical particles (SpNp)	30
2.4.3 Siliceous mesocellular foam (SCM Foam)	31
2.4.4 Silica functionalization	32
2.5 Fluorescence Principles	33
2.5.1 Fluorescence characteristics	34
2.6 Fluorescent anisotropy	35
2.7 Software used	38
3 References	39
Chapter 4: Improving the activity and stability of CLEAs and Magnetic CLEAs with epoxides.	41
Abstract	43
1 Introduction	44
2 Materials and Methods	47
2.1 Materials	47
2.2 Aldolase production	47
2.3 CLEAs synthesis	48
2.4 Mathematical optimization method	49
2.5 Tomography microscopy of epoxy-CLEAs	50
2.6 Stability assay	50

2.7 Magnetic CLEAs synthesis	50
2.8 Aldolase activity measurements	51
3 Results and Discussion	52
3.1 Preliminary assays	52
3.2 Mathematical optimization	53
3.3 Validation of the model	60
3.4 Tomography microscopy of epoxy-CLEAs	61
3.5 Stability assay	64
3.6 Magnetic CLEAs	64
4 Conclusions	69
5 References	71
Chapter 5: Epoxide-based immobilization of Laccase inside mesocellular foam as green catalyst for Phenoxazinone synthesis.	75
Abstract	77
1 Introduction	78
2 Materials and Methods	81
2.1 Materials	81
2.2 Preliminary assays	81
2.3 Siliceous support preparation	81
2.4 Experimental design and statistical analyses	82
2.5 Laccase immobilization procedures	82
2.6 Laccase activity test	83
2.7 Laccase mediated phenoxazine synthesis	83
2.8 Free and immobilized laccase kinetic studies for AHBS substrate	84

2.9 Biocatalyst temperature stability and reusability	84
3 Results and Discussion	86
3.1 Preliminary assays	86
3.2 Optimization of Laccase immobilization	87
3.3 Phenoxazinone compound synthesis	92
3.4 Kinetic studies	95
3.4.1 Optimum pH and temperature	95
3.4.2 Kinetic parameters	96
3.5 Biocatalyst thermal stability and reusability	96
4 Conclusions	98
5 References	101
Chapter 6: Cinnabarinic acid an active compound: Characterization and Synthesis by immobilized <i>Trametes versicolor</i> laccase.	103
Abstract	105
1 Introduction	106
2 Materials and Methods	111
2.1 Materials	111
2.2 Methods	111
2.2.1 Siliceous support preparation	111
2.2.2 Laccase immobilization procedure	112
2.2.3 Laccase activity test	112
2.2.4 Laccase mediated cinnabarinic acid synthesis	112
2.2.5 Cinnabarinic acid formation with periodate	113
2.2.6 Coleman analysis	113

2.2.7 3-Hydroxyanthranilic acid and Cinnabarinic acid pH dependence studies	113
2.2.8 Free and immobilized laccase kinetic studies for 3-Hydroxyanthranilic acid	114
2.2.9 Biocatalyst reusability	115
2.2.10 Cinnabarinic acid HPLC analysis	115
3 Results and Discussion	116
3.1 Laccase mediated cinnabarinic acid synthesis	116
3.2 3-Hydroxyanthranilic and cinnabarinic acid pH dependence studies	121
3.3 Laccase immobilization	123
3.4 Free and immobilized laccase kinetic studies for 3-Hydroxyanthranilic acid	124
3.5 Biocatalyst reusability	126
3.6 HPLC analysis	127
4 Conclusions	129
Supplementary Material	130
5 References	135
Chapter 7: Functionalization of magnetic particles by means of nucleophilic substitution of organic halides.	139
Abstract	141
1 Introduction	142
2 Materials and Methods	144
2.1 Materials	144
2.2 Methods	144
2.2.1 Magnetic Nanoparticles preparation.	144

2.2.2 Magnetic nanoparticles Epichlorohydrin Surface functionalization	144
2.2.3 Magnetic nanoparticles 1, 3, 5 Trichloro 2, 4, 6 triazine Surface functionalization	145
2.2.4 TEM characterization	145
2.2.5 Titration of epoxy groups in the surface	145
2.2.6 Lipase immobilization with epoxy magnetic nanoparticles	146
2.2.7 Amine surface modification	146
2.2.8 Amine determination with Naphthoquinone sulfonate	147
3 Results and Discussion	148
3.1 Organic halides magnetic particles direct functionalization	148
3.2 Titration of surface epoxides	150
3.3 Lipase immobilization with epoxy magnetic nanoparticles	150
3.4 Amine surface modification	151
4 Conclusions	153
5 References	154
Chapter 8: Magnetic Nanometric Lipase for solvent free Ester Synthesis.	157
Abstract	159
1 Introduction	160
2 Materials and Methods	163
2.1 Materials	163

2.2 Methods	163
2.2.1 Magnetic Nanoparticles preparation.	163
2.2.2 Magnetic Nanoparticles surface activation.	163
2.2.3 Lipase immobilization procedures	164
2.2.4 Lipase activity test	165
2.2.5 Benzyl acetate Synthesis	165
2.2.5.1 Vinyl acetate and benzyl alcohol molar relation	166
2.2.5.2 Temperature profile	166
2.2.5.3 Minimal water amount	166
2.2.5.4 Kinetic parameters	166
2.2.6 Biocatalyst recycle	167
2.2.7 HPLC analysis of Benzyl acetate	168
3 Results and Discussion	169
3.1 Magnetic nanoparticles preparation and fuctionalization	169
3.2 Lipase immobilization	166
3.3 Benzyl acetate synthesis	171
3.3.1 Vinyl acetate and benzyl alcohol molar rate	171
3.3.2 Temperature profile	173
3.3.3 Minimal water amount	173
3.3.4 Kinetic study	175
3.4 Biocatalyst reuse	176
4 Conclusions	177
5 References	178

Chapter 9: An improved method to measure Lipase activity in aqueous media.	181
Abstract	183
1 Introduction	184
2 Materials and methods	186
2.1 Materials	186
2.2 Methods	186
2.2.1 <i>p</i> -nitrophenol calibration curve	186
2.2.2 Preparation of Substrate Stock solutions	186
2.2.3 Lipase activity assay optimization	187
2.2.4 Lipase general activity assay	187
2.2.5 Effect of enzyme mass	188
2.2.6 Optimization of ethylene glycol	188
2.2.7 Temperature and pH optimization	188
2.2.8 Kinetic studies	188
2.2.9 Effect of additives	189
3 Results and Discussion	190
3.1 <i>p</i> -nitrophenol calibration curve	190
3.2 Enzyme mass	190
3.3 Ethylene glycol effect	191
3.4 Temperature and pH profiles	192
3.5 Kinetic parameters	193
3.6 Effect of additives	194
4 Conclusions	196
5 References	197

Chapter 10: Absorbance and Fluorescence studies of Anthranilic, 3-Hydroxyanthranilic and Cinnabarinic Acids.	199
Abstract	201
1 Introduction	202
2 Materials and Methods	204
2.1 Materials	204
2.2 Methods	204
2.2.1 Relationship between the absorbance of anthranilic, hydroxyanthranilic and cinnabarinic acids and pH	204
2.2.2 Water affinity of anthranilic, hydroxyanthranilic and cinnabarinic acids	204
2.2.3 Fluorescence spectra of anthranilic, 3-hydroxyanthranilic and cinnabarinic acids	205
2.2.4 Fluorescence of anthranilic, 3-hydroxyanthranilic and cinnabarinic acids with different pH	205
2.2.5 Fluorescence of anthranilic, 3-hydroxyanthranilic and cinnabarinic acids in different solvents	206
3 Results and Discussion	207
3.1 Relationship between the absorbance of anthranilic, 3-hydroxyanthranilic and cinnabarinic acids and pH	207
3.2 Water affinity of anthranilic, hydroxyanthranilic and cinnabarinic acids	209

Abbreviations Index

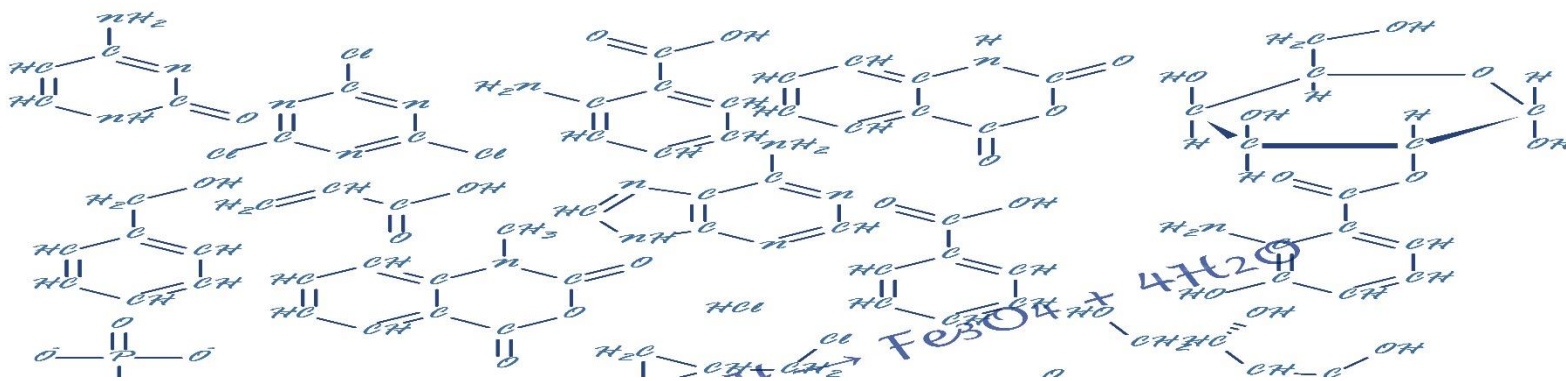
Abb

3-HAA	3 Hydroxyanthranilic acid
AA	Anthranilic acid
Abs	Absorbance
ABTS	2-2'-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid
AHBS	3-amino-4-hydroxybenzene sulfonate
ANOVA	Analysis of Variance
APS	Ammonium persulfate
APTES	(3-Aminopropyl) triethoxysilane
BSA	Bovine Serum Albumin
C18-FL	Octadecyl-fluorescein
C18-MI	Octadecyl-methylisatoic
CCB	Colloidal Coomassie Blue
CLEAs	Cross-linked enzyme aggregates
CLECs	Cross-linked enzyme crystals
DTAF	5-([4, 6-Dichlorotriazin-2-yl] amino) fluorescein
df	Degrees of freedom
DEGDE	Diethylene glycol diglycidyl ether
DMSO	Dimethylsulfoxide
DRCC	Design rotational central composite
DOE	Design of experiments
<i>E. coli</i>	<i>Escherichia coli</i>
Eq.	Equation

F.I	Fluorescent intensity
FIAsH	4',5'-bis(1,3, 2-dithioarsolan-2-yl)fluorescein
FsNp	fumed silica nanoparticles
FPLC	Fast protein liquid chromatography
GDE	Glycerol diglycidyl ether
HIS	Histidine
HPLC	High performance liquid chromatography
IOD	Integrated Optical Density
IPTG	Isopropyl β -D-1-thiogalactopyranoside
LOF	Lack of fit
NAL	Acetilneuraminiclyase
NADH	Nicotinamide adenine dinucleotide
Neu5 Ac	n-acetyl-D-neuraminic acid
NTA	Nitrilotriacetic acid
NTA-FL	Nitrilotriacetic-fluorescein
NTA-MI	Nitrilotriacetic-methylisatoic
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PEG	Polyethylene glycol
ROS	Reactive Oxygen Species
RSM	Response surface methodology
SS	Sum of Squares
SDS	Sodium dodecyl sulfate
SMC foam	siliceous mesocellular foam
spNp	spherical particles
TEM	Transmission electron microscopy

TEMED	Tetramethylethylenediamine
TMPTGE	trimethylolpropane triglycidyl ether
TEOS	tetraethyl orthosilicate
UV	ultraviolet

Chapter 1



Introduction

Chemical Modification of Proteins

Overview

1 Chemical modification of proteins

The chemical modification of proteins started many years before to any significant studies in the field, as the use of formaldehyde in the tannin of leather process or ancient formulations (Streitwieser et al. 1992) without understanding the chemical reactions involved. The interest of techniques and reagents to modify proteins surged in the 80s and it had been expanded till now. An increasing number of specialized reagents have been described: affinity labels, specific site directed reagents or chemical compounds that reacted predominantly with one particular kind of amino acid side chain (i.e. thiol, amine, hydroxyl or carboxyl) and others that reacts nonspecifically with different side chains (Means & Feeney 1990).

Methods and reagents had been development to decrease the immunogenicity (De Groot & Scott 2007), increase the protein absorbance, to attach fluorescent probes (Giepmans et al. 2006), to introduce metal ions or magnetic nanoparticles, to include biospecific recognition groups (Cronan 1990) or to introduce intermolecular cross-links to produce protein-protein conjugates (Silva et al. 2004).

Chemical modification of enzymes is a powerful complementary technique to mutagenesis and directed evolution to produce tailor made enzymes. Glutaraldehyde cross-linking to form insoluble enzyme crystals, PEGylation to make them more soluble in organic solvents or the incorporation of cofactors are common techniques to enhance the functionality of the enzymes (DeSantis & Jones 1999).

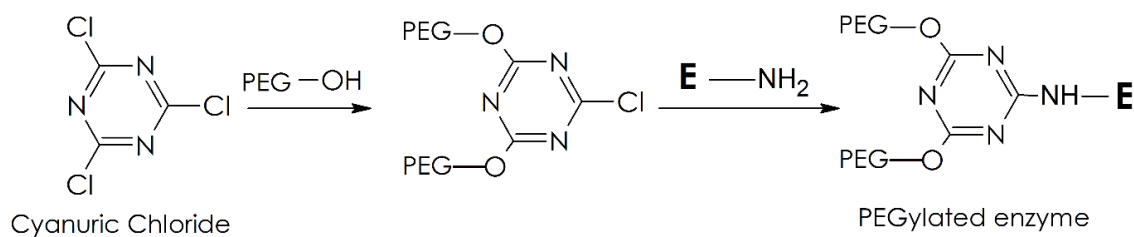
Protein cross-linked had been used to increase the stability of enzymes, particularly in organic solvents. The stabilization is produced due inter-molecular cross-linking with bifunctional or polyfunctional reagents. Glutaraldehyde was the first bifunctional reagent used to cross-link enzymes (Quiocho & Richards 1964) and produce cross-linked enzyme crystals (CLECs). These crystals were insoluble and retained catalytic activity. Other bifunctional reagents had been tested and used to

cross-link enzymes, the most outstanding cross-linking reagents were summarized in Table 1.

Table 1: Protein crosslinked bifunctional reagents.

Reagent	Reactivity Description	Proteins cross-linked	Ref.
glutaraldehyde	Reaction with amino groups forming a Schiff base. That can be reduced to secondary amine using Borohydride	Lipases Lyases Oxidoreductases Proteases	(Sheldon 2011)
Dimethyl suberimidate (DMS)	Reacts with amino groups at pH 8.0-10.0, to form amidine bonds, reversible at high pH.	Aldolase Tryptophan synthetase	(Davies & Stark 1970)
Disuccinimidyl suberate (DSS)	React with primary amines at pH 7.0-9.0 to form stable amide bonds, releasing N-hydroxysuccinimide.	Alkaline phosphatase	(Leary et al. 1983)
Bismaleimido-hexane (BMH)	Reacts with sulfhydryls at pH 6.5-7.5 to form thioethers. Although it can react with amines at pH > 8.0, but the reaction rate is 1000 times slower than with thiols.	γ -Crystallins Camodulin	(Asherie et al. 1998) (Persechini & Kretsinger 1988)
2-iminothiolane (Traut's reagent)	Reacts with amines at pH 7.0-10.0. Also, at high pH reacts with hydroxyl groups, however the reaction rate is slower.	Ribosomal subunits	(Uchiumi et al. 1985)

PEGylation of proteins is a chemical modification of proteins used to enhance their solubility in organic solvents and to reduce their antigenicity (for protein preparations to be used *in vivo*). A normal procedure consisted in the modification of cyanuric chloride with polyethylene glycol molecules, then react the modified cyanuric chloride with the desired protein, as depicted in Scheme 1.



Scheme 1: Enzyme modification by PEGylation.

Some examples of enzyme modification with polyethylene glycols are lipase from *Candida rugosa* (Hernaiz et al. 1997), catalase (Hyoudou et al. 2006) or cytochrome C (Tinoco & Vazquez-Duhalt 1998). PEG-catalase avoided tumor metastasis by detoxification of ROS (reactive oxygen species) in mice footpad tumors (Hyoudou et al. 2006).

Inclusion of small moieties in the protein structures has proven useful, as the incorporation of D-glucosamine in RNase A. The modified enzyme showed better thermal stability than the native enzyme (Baek & Vijayalakshmi 1997).

2 Enzyme immobilization overview

The use of enzyme immobilization starts in the 1800 with the production of vinegar. Usually an alcohol solution was trickled over bacteria overgrown wood shavings. Along with the water clarification were the first examples of immobilized enzymes used in transformation process. Later in 1960 several immobilization technologies and uses were developed, such as the production of L-aminoacids or the isomerization of glucose. From the 80s till now there is great interest in the improvement and implementation of immobilized enzymes and whole cells in industrial processes, to make them environmentally acceptable and economically sustainable.

There are several reasons to use enzymes or in particular immobilized enzymes in synthesis processes. In addition to improving the handling of enzymes, the main

advantages of this enzyme preparations are: the easily remove of the proteins from the final product, the upgraded stability, the higher selectivity and activity yield, the improved performance in organic solvents and the possible reuse of the biocatalyst (Fig 2).

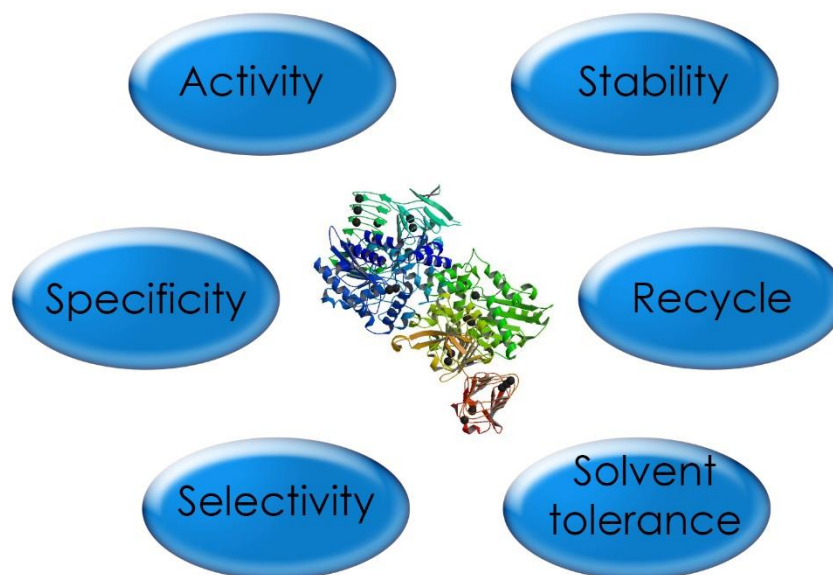


Fig 2: Enzyme as biocatalyst for chemical reactions overview.

Facilitating the separation of the enzymes from the reaction simplifies the purification of the final product and is compatible with a reliable and efficient reaction technology. On the other hand, the reuse of enzymes provides a reduction in production costs, which is often an essential requirement to implement an enzyme-catalyzed process.

The properties of the immobilized enzyme preparations are governed by the properties of both the enzyme and the carrier material. The specific interaction of the enzyme with the support results in an immobilized enzyme with different chemical, biochemical, mechanical and kinetic properties. The most important parameters to consider when immobilizing enzymes are: the biochemical and kinetic properties of the enzyme, the physical-chemical and mechanical properties of the support, the type

of immobilization, the stability and productivity of the enzyme Immobilized and the effects of transfer of matter derived from the immobilization.

The immobilization procedures can be arranged in three groups (Fig 3): cross-linking (as known as carrier free), carrier binding immobilization and entrapment.

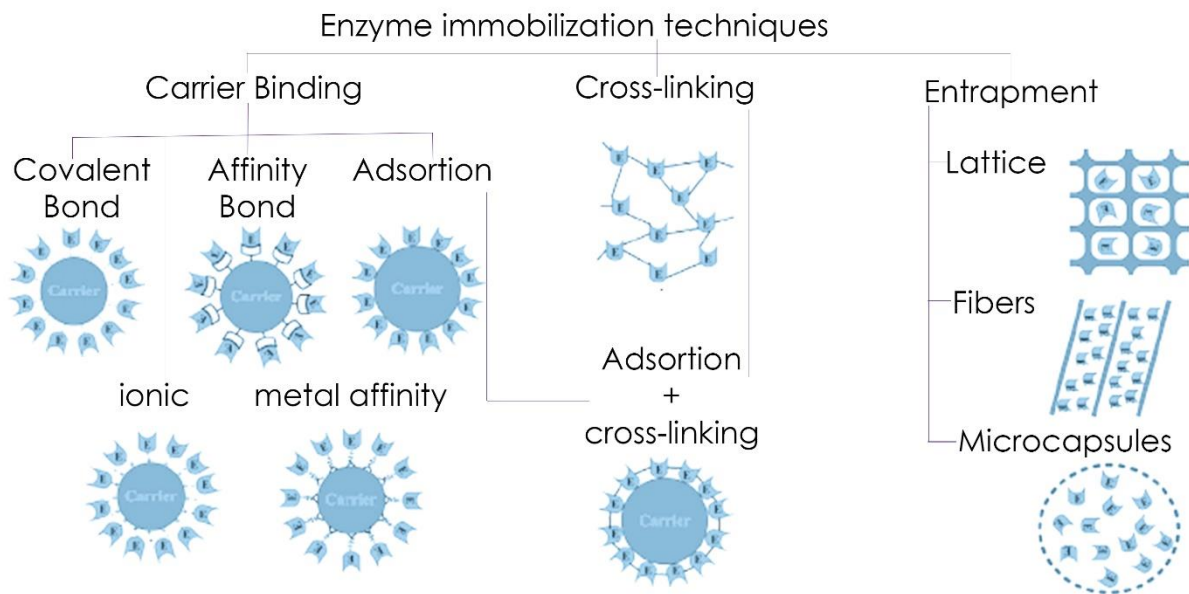


Fig 3: Enzyme immobilization methods.

2.1 Immobilization by Cross-linking (carrier free immobilization)

The addition of salts or solvents miscible in water or organic nonionic polymers to aqueous solutions of proteins leads to their precipitation in the form of physical aggregates. The aggregates are bonded non-covalently and maintain their tertiary structure, i.e. without denaturing the protein. Therefore, the subsequent reaction of these physical aggregates, with a suitable bifunctional reagent, makes them permanently insoluble while maintaining their superstructure organized, and their catalytic activity, as shown Fig 4. Since the precipitation of enzymes from an aqueous medium, by the addition of salts (ammonium sulfate) or polyethylene glycols, is often used to purify enzymes, the CLEAs (cross-linked enzyme aggregates) methodology

essentially combines purification and immobilization in a single operation (Sheldon et al. 2003).

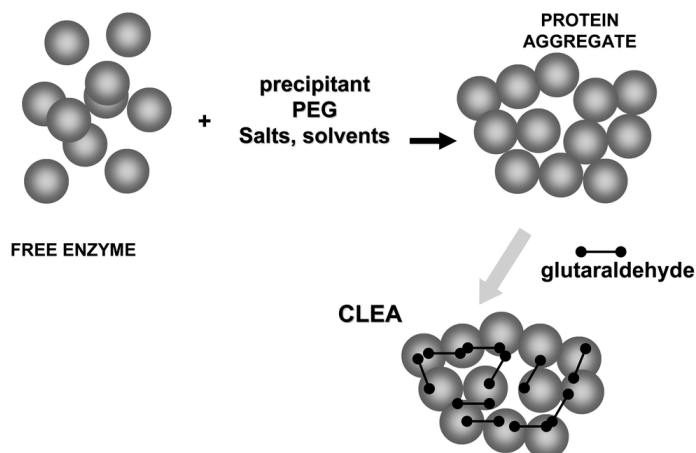


Fig 4: CLEAs technology overview.

Using water-in-oil emulsions cross-linked enzymes (Spherezymes) (Brady et al. 2008) can be also prepared. Through addition of protein cross-linking agents to a water-in-oil emulsion of an aqueous enzyme solution, structured self-immobilised spherical enzyme particles of lipase were formed, as depicted in Fig 5 lipases are situated on the emulsion interphase, due its hydrophobic nature. This technique has the advantage over the CLEAs of being able to control the size of the aggregates, by controlling the parameters of the emulsion (agitation, surfactants, amount of water and oil).

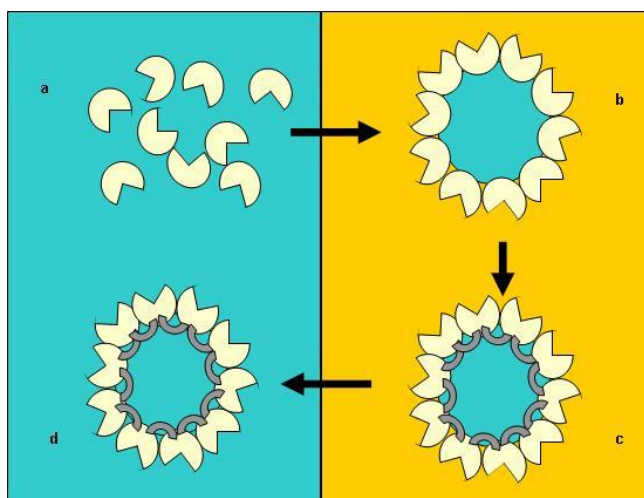


Fig 5: Spherezymes cross-linking method scheme.

2.2 Carrier bond immobilization

2.2.1 Binding techniques

The carrier bond immobilization involves the use of a solid support, on which enzymes are attached. Supports can be of organic or inorganic nature, but also a composite of both. The enzymes can be adsorbed, affinity bonded or covalent bonded onto the support surface.

Enzyme adsorption is the easiest method to bind enzymes on supports. The enzyme binds to the material due hydrophobic interactions, cation/anion exchanges or Van der Waals forces. An Example of this technique is the immobilization of *Aspergillus niger* lipases via adsorption on C8 magnetic particles (Hernández-García et al. 2014). The adsorbed lipase showed more activity towards p-nitrophenyl decanoate ($17.5 \mu\text{mol min}^{-1} \text{mg}^{-1}$) than the free crude extract ($2 \mu\text{mol min}^{-1} \text{mg}^{-1}$) indication of a purification process that the lipase undergoes when adsorbed on the particles and its adsorption in a more active structural conformation. Other example was the adsorption of commercial laccase, DeniLite IIS, on green coconut fibers (Cristóvão et al. 2011). The immobilized enzyme was used for reactive dyes degradation, such as RY15, RR239 or RB5.

Covalent binding is the result of the reaction between the support groups and the enzyme side chain aminoacids, like arginine, lysine, aspartic acid or histidine. The attachment can be achieved by direct reaction of a reactive group in the support and the side chains of the protein or using a cross-linking reagent to bond the enzyme to the support. A comparative example of the immobilization of alkaline protease from *Bacillus mycoides* on cyanogen bromide activate sepharose and chitosan modified with glutaraldehyde (Abdel-Naby et al. 1998). The chitosan support activated with glutaraldehyde showed better immobilization yield (77.41 %) than the activated sepharose (63.91 %).

Affinity immobilization exploits the specificity of the enzyme toward a modified support. Either the support is prepared with an affinity ligand for a target enzyme, or the enzyme is modified with a motif with affinity toward the support. Supports modified with nickel charged nitrilotriacetic acid permitted the purification and immobilization of histidine-tagged proteins in one step. The technique had been used for acetylcholinesterase immobilization in an organophosphorus insecticides sensor (Andreescu et al. 2002). Other extended method is the coupling of the support with avidin to attach biotin modified proteins. Biotinylated glucose oxidase had been deposited on avidin monolayers and used as glucose sensor (Hoshi et al. 1995).

2.2.2 Materials for supports preparation

There are many different examples of materials used to immobilize enzymes in the bibliography (Datta et al. 2013). The supports are classify due its nature: natural or synthetic materials.

Between the natural materials, polymers like chitin, chitosan, or sepharose are widely used in enzyme immobilization procedures.

Chitin is a polysaccharide composed of β (1 \rightarrow 4)-linked 2-acetamido-2-deoxy- β -D-glucose (N-acetylglucosamine). Chitin is found in invertebrate's exoskeleton, like crustacean shells. Similar chitosan is a linear polymer formed of α (1 \rightarrow 4)-linked 2-amino-2-deoxy- β -D-glucopyranose. They had been applied in cosmetic, water treatment, food processing and more (Dutta et al. 2004). In addition chitin and chitosan had been used to immobilize plenty of enzymes, such as lipase, glucose isomerase, invertase, raffinase of papain (Krajewska 2004).

Sepharose is a commercial prepared (GE Healthcare) agarose crosslinked beads. The agarose polymer is obtained from sea weeds. The most commonly use of this polymer is the chromatographic separation of biomolecules as well as enzyme immobilization (Ahmed 2004).

Chapter 1

On the other hand there are the synthetic polymers and inorganic materials as immobilization support. In the bibliography a wide diversity of inorganic materials used as support can be found. Outstanding the siliceous materials, the zeolites or methacrylamide copolymers.

Siliceous supports are prepared by condensation of silica precursors. Siliceous supports can be prepared in different sizes, from nanometric to micrometric sizes, can be porous or solid, and can be modified with several reactive groups as amines, thiols or oxirane. Glucose oxidase was immobilized inside mesoporous silica support by glutaraldehyde crosslinking. The immobilized enzyme was used as glucose sensor (Wilson et al. 2000).

Zeolites (molecular sieves) are microporous crystalline aluminosilicate materials used as adsorbents and as catalyst. These materials had also been used as enzyme immobilization supports. As example cutinase from *Fusarium solani pisi* was immobilized by adsorption on several zeolites and its activity towards the alcoholysis reaction of butyl acetate with hexanol, in isooctane measured (Serralha et al. 1998). Glucose oxidase was also immobilized in zeolite to construct an amperometric glucose biosensor (Liu et al. 1997).

Eupergit® is a commercial support well known in enzyme immobilization field. The support contains oxirane motifs in the surface, which permits the covalent attachment of enzymes. Lipase from *P. fluorescens*, glucose oxidase, tripsin, phosphodiesterase and more (Mateo et al. 2007) had been covalent immobilized on this commercial support.

In addition of these immobilization materials the incorporation of magnetic particles to the immobilization supports had been developed. Using supports that contain a magnetic core facilitates the separation of the enzymes from the reaction medium and its recycle (Halling & Dunnill 1980). Galactose oxidase from *Dactylium*

dendroides and neuraminidase from *Clostridium perfringens* had been successfully immobilized on magnetic poly(HEMA-co-EDMA) microspheres (Bilková et al. 2002).

2.3 Immobilization by entrapment

In this technique enzymes are physically entrapped inside a porous matrix. The pore size is modulated to avoid enzyme loss, however the pores sized must be wider enough to permit the cross of substrates and products. The most usually used matrixes to entrap enzymes are: polyacrylamide, cellulose, agar, gelatin, or alginate.

The entrapment can be divided in: gel entrapment, where the enzymes are inside a gel matrix, inclusion in fibers: the enzymes are supported in fibers, and inclusion in microcapsules: the enzymes are trapped in monomeric microcapsules, like calcium-alginate spheres as the showed in Fig 5. Proteases from *Bacillus subtilis* were trapped in calcium-alginate beads (Anwar et al. 2009). The immobilized enzymes showed better reusability than the free enzymes.



Fig 5: Calcium-alginate beads

The process of entrapment via sol-gel is based on the ability of certain chemical precursors to form metallic or semi-metallic oxides via hydrolysis. Hydrolysis of a precursor (silicic acid, silicic alkoxide) in an aqueous medium produces a sol of soluble oligomers, which are capable of polymerizing to give a hydrogel. The controlled extraction or drying of water from the hydrogel results in a matrix of silicon oxides, with a Si-O-Si type skeleton, with microstructured pores, where biomolecules can be

confined. Polyvinyl alcohol hydrogels (PVA) LentiKats[®] (Fig 6) has also been developed (Schlieker & Vorlop 2006) to immobilize *Clostridium butyricum* whole cells.

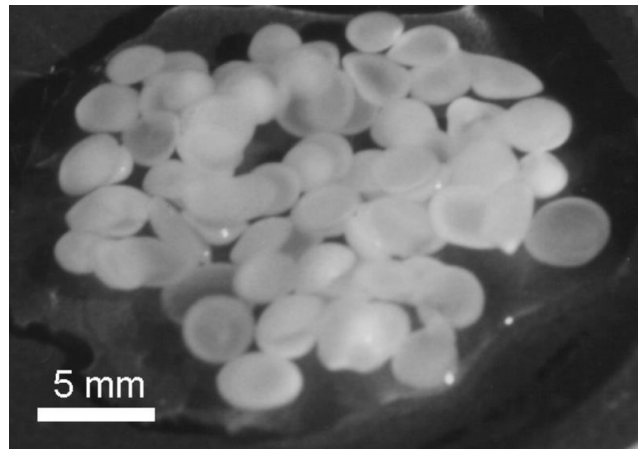


Fig 6: LentiKats[®] images.

3 Protein fluorescent labeling

Fluorescence is the property of some substances to emit light when irradiated. This property had been exploited for imaging and visualization in molecular and cell biology, as shown in Fig 7. It had been used from molecules to whole cells.

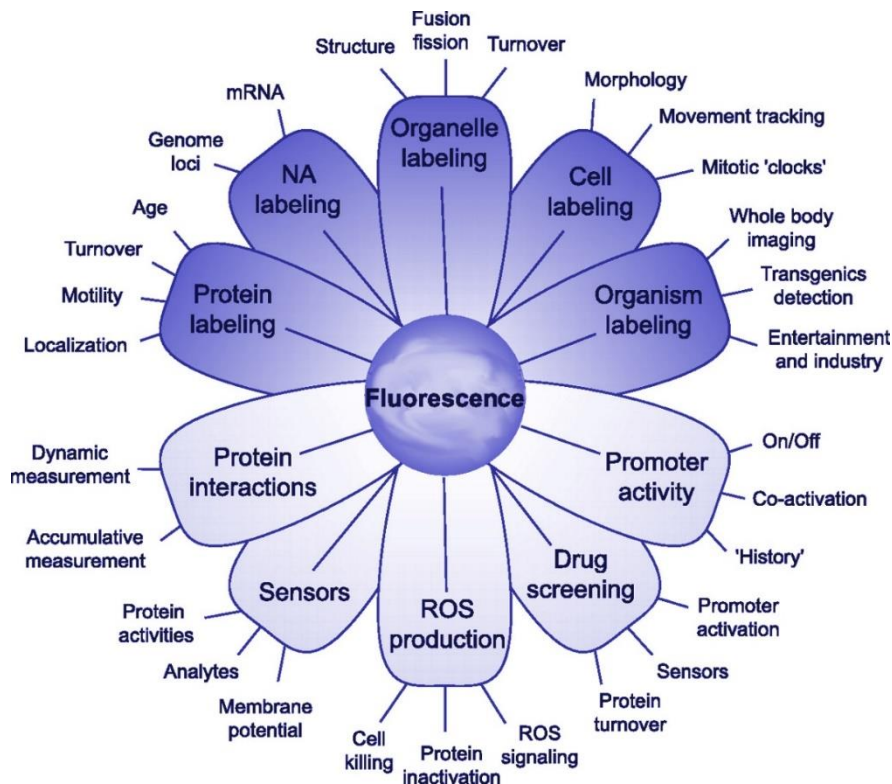


Fig 7: Fluorescence uses in molecular and cell biology.

3.1 Fluorophores for labeling

The fluorophores (molecules with fluorescence) used are divided in: organic fluorescent dyes and fluorescent proteins (Giepmans et al. 2006).

Hundreds of organic dyes had been developed and tested to be used in fluorescent imaging. The dyes properties as brightness, Stokes shift or quantum yield had been optimized for these applications, being the most used fluorescein and rhodamine. Usually this molecules were coupled to an antibody to most of the applications (immuno-labeling). However the labeling can be also achieved directly, by chemical reaction of the fluorophore and the protein or using affinity tags, whereas the fluorescent labeled molecule had affinity toward a tagged protein. The first is a universal labeling method, while the affinity will only label the marked protein making the method very selective. An example of immune-labeling is the detection of forensic fingerprints in different materials. The fingerprints were coupled with an antibody which is marked with a fluorescent labeled secondary antibody (van Dam et al. 2014) as showed in Fig 8.

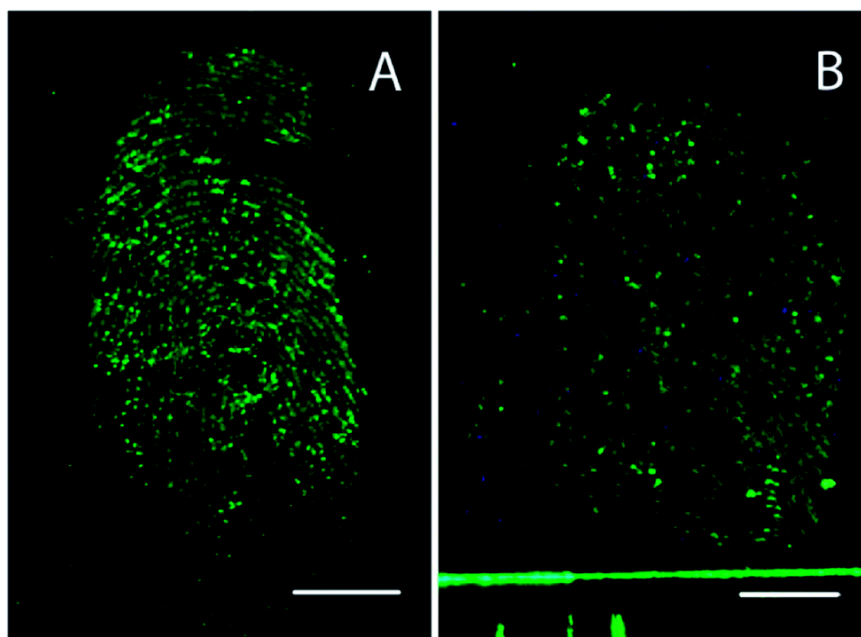


Fig 8: Immuno-labeling detection of fingerprints

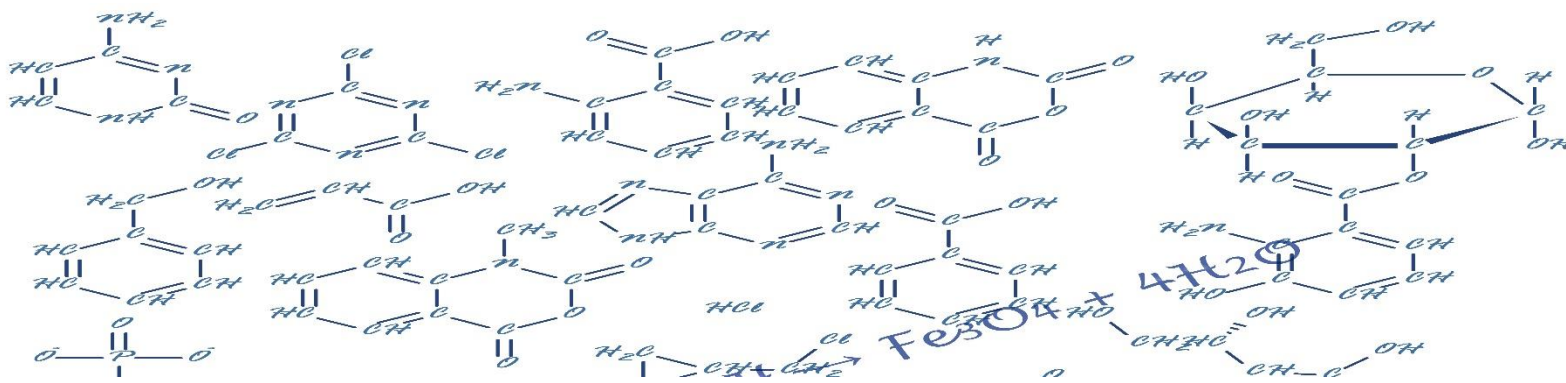
Chapter 1

Fluorescent proteins were a revolution in cell biology imaging, in special when the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Fig 9) was discovered (Shimomura et al. 1962), cloned and sobreexpressed (Cormier 1992). GFP can be expressed alone or fused with other proteins and results in visible fluorescence without the need of cofactors. Homologous of GFP with different colors and properties had been obtained mostly from marine coelenterates, or genetic mutations of GFP as azurite (Mena et al. 2006) or YFP (yellow fluorescent protein) (Hansen & O'shea 2013).



Fig 9: Image of the jellyfish *Aequorea Victoria*

Chapter 2



Objectives

Objectives

The main objective of this thesis is the development of new techniques and reagents to modify proteins and its applications in enzyme immobilization, catalysis and fluorescent labeling. This main objective could be divided in the following sub-objectives:

1 Improve the carrier-free immobilization technology.

1.1 Research for economically sustainable reagents to replace glutaraldehyde as crosslinking reagents.

1.2 Optimization and production of aldolase epoxy-CLEAs, using statistical approach.

1.3 Characterization of the aldolase epoxy-CLEAs.

2 Preparation of a laccase biocatalyst based in siliceous particles, and its application in oxidation reactions, such as phenoxazines production.

2.1 Preparation and evaluation of several siliceous support.

2.2 Laccase immobilization procedure optimization using design of experiments and response surface.

2.3 Application to AHBS production.

2.4 Application to cinnabaric acid synthesis and characterization.

3 Development of a magnetic lipase and its use in solvent free synthesis.

3.1 Improve the magnetic nanoparticles surface modification method.

3.2 Lipase covalent attachment to the nanoparticles.

3.3 Improve the lipase hydrolytic activity measurement method in aqueous medium.

3.4 Solvent free synthesis of benzyl acetate with magnetic lipase.

4 Research for low cost fluorophores and its applications in protein labeling, quantification and gel stain.

4.1 Evaluation of isatoic acid anhydride analogous as potential fluorophores.

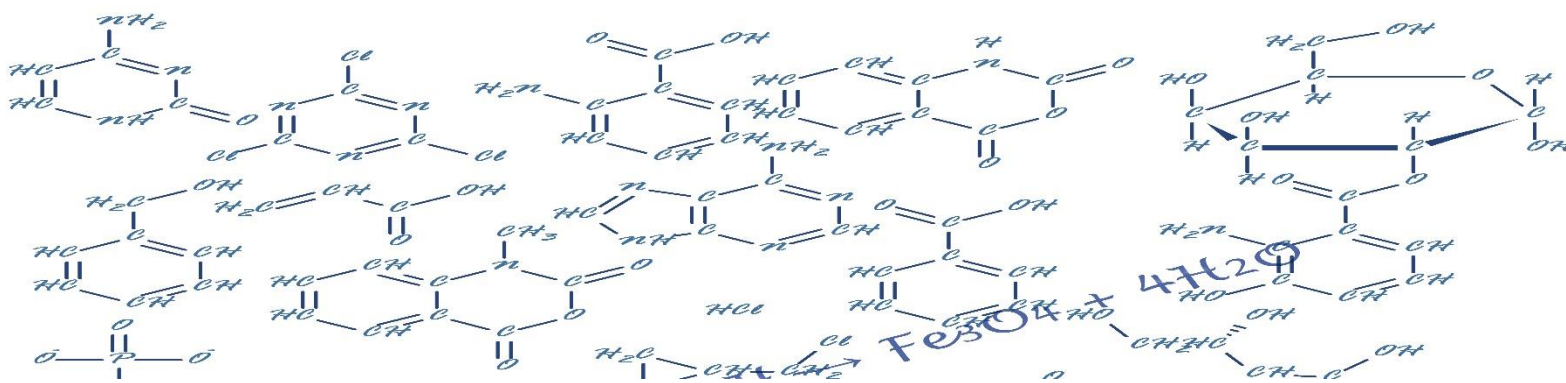
4.2 Preparation of a triazine activated fluorescein.

4.3 Covalent protein labeling and gel staining with isatoic anhydride and fluorescein.

4.4 Synthesis of fluorescent-NTA conjugates for sensitive, specific detection of Polyhistidine-Tagged Proteins.

4.5 Synthesis of hydrophobic derivatives of isatoic anhydride and triazine fluorescein, applications in gel staining protein quantification and cell signaling.

Chapter 3



Materials and Methods overview.

This is an overview of the most common methods used in the Thesis, such as enzyme quantification methods or SDS-PAGE. The specific methods used in every chapter will be described and detailed in each chapter Materials and Methods section.

1 Materials

1.1 Chemical reagents

The materials used in each chapter will be summarized in the epigraph 2. Materials and Method for the chapter.

1.2 Proteins

The Neu5Ac Aldolase was cloned and purified in our laboratory as related García-García et al (García-García et al. 2014), this enzyme was used in the Chapters 4, 11, 12 and 13.

Laccase from *Trametes versicolor* (13.6 U/mg, 1 U corresponds to the amount of enzyme which converts 1 μ mol catechol per minute at pH 4.5 and 25 °C) used in the Chapters 5 and 6 was purchased from Sigma Aldrich (Sigma-Aldrich Quimica SL Madrid, Spain). The protein (5 mg) was dissolved in 1 mL sodium phosphate buffer (20 mM, pH 7.0) and precipitated in 10 mL of cold acetone for 10 minutes in ice. Then centrifuged 10 min 7000 rpm, and the acetone supernatant discarded. Laccase pellet was left to dry for 5-10 minutes and resuspended in buffer.

Lipases from *Mucor javanicus* and *Pseudomonas fluorescens* used in Chapter 7, 8 and 9, were obtained from Sigma Aldrich (Sigma-Aldrich Quimica SL Madrid, Spain), and used without further purification.

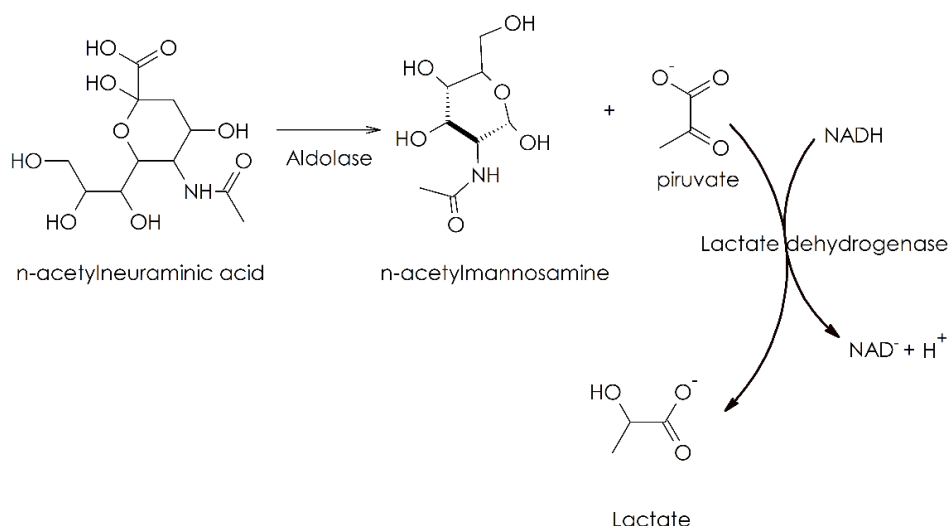
BSA (bovine serum albumin), Lysozyme from egg white, Trypsin, and protein marker (SigmaMarker™) wide range 6.5-200 kDa used in the Chapters 11, 12 and 13 were obtained from Sigma Aldrich (Sigma-Aldrich Quimica SL Madrid, Spain).

2 Methods

2.1 Enzymes general activity test

2.1.1 Aldolase activity measurements

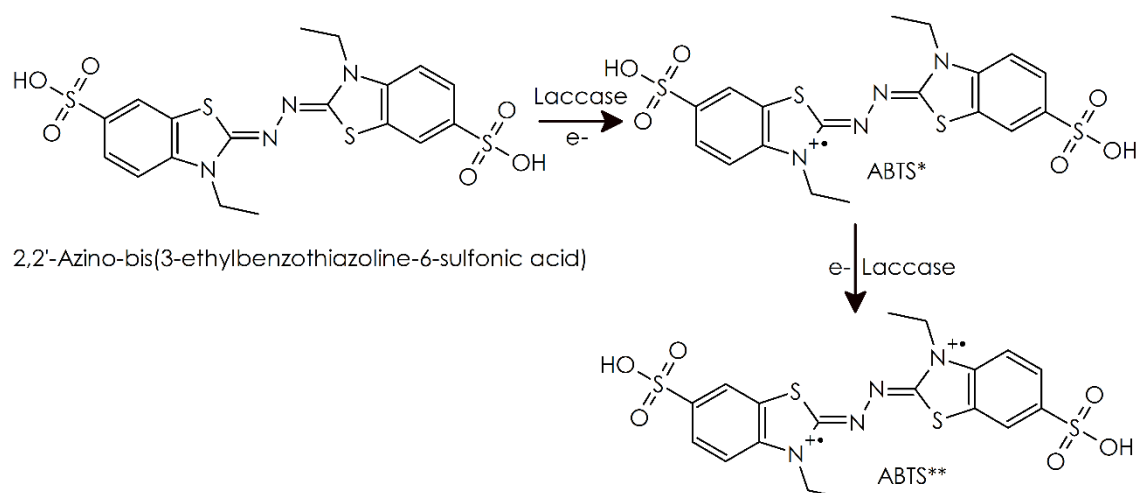
Enzyme activity was determined spectrophotometrically with a coupling reaction following Scheme 1, measuring the decrease of absorbance at 340 nm corresponding the NADH oxidation to NAD by L-lactate dehydrogenase. The aldolase produces pyruvate from the Neu5Ac cleavage, then the pyruvate is transformed to lactate by L-lactate dehydrogenase oxidizing NADH to NAD. All the measurements were made in sodium phosphate 20 mM pH 7.0 and 37 °C.



Scheme 1: Aldolase activity test

2.1.2 Laccase activity test

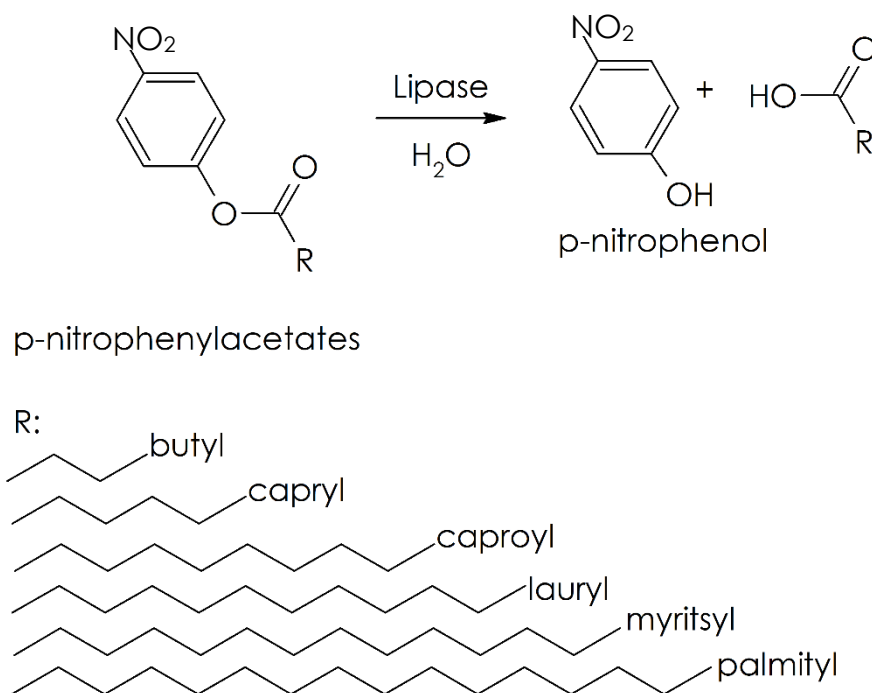
2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) was used as substrate to determine laccase activity as Scheme 2 illustrates. Fifty microliters of ABTS (0.534 mM) prepared in acetate buffer (100 mM, pH 3.0) was added to 50 μ L of laccase sample and 900 μ L of the same buffer, in 1 mL spectrophotometric cuvette. The ABTS oxidation (formation of a green compound) was monitored by measuring the absorbance at 420 nm (ϵ 36000 M⁻¹ cm⁻¹).



Scheme 2: Laccase activity test

2.1.3 Lipase activity test

Lipase activity was measured with *p*-nitrophenyl ester substrates like proposed in Scheme 3, following our improved method. The *p*-nitrophenyl substrates stock solutions (500 mM), were adjusted to 25 mM with tris/acetate 50 mM pH 6.5, being the final concentration of ethylene glycol 5 %. In a 1 mL spectrophotometer cuvette 880 μ L of tris/acetate buffer 50 mM pH 6.5 with 5 % of ethylene glycol and 100 μ L of the prepared substrate were added, reaction was started by adding 20 μ L of lipase (1 mg/mL). The formation of the yellow compound 4-nitrophenol was measured at 405 nm.



Scheme 3: Lipase activity test

2.2 Protein quantification

Two methods were used for protein quantification, Bradford and Bicinchoninic methods. Bradford was the overall standard method, while Bicinchoninic was used when surfactants or detergents are present as they interfered with Bradford.

2.2.1 Bradford method

Bradford method (Bradford 1976) was adjusted to 96 well plate, 5 μL of sample or protein standard (BSA) was mixed with 250 μL of Bradford reagent (BioRad) and incubated for 15 minutes at room temperature. Plates were read in a Synergy microplate reader (BioTek Instruments, Inc.) at 595 nm. Bradford method is lineal between 0.2-2 mg/mL of protein, assays were replicated trice and the final result was the mean of the obtained values.

2.2.2 Bicinchoninic method

Bicinchoninic acid method (Smith et al. 1985) was also used in 96 plate well, 5 mL of Bicinchoninic acid solution (Sigma Aldrich) was mixed with 0.1 mL of 4 % w/v copper (II) sulfate pentahydrate to prepare working solution, 200 μ L of the solution was mixed with 25 μ L of sample or BSA standard. The plate was incubated at room temperature for 2 hours and the absorbance was measured at 562 nm. The method is linear in the range between 0.02 to 20 mg/mL of sample.

2.3 Protein Electrophoresis

2.3.1 Acrylamide SDS-PAGE gels

Acrylamide denaturalizing gels (SDS-PAGE) (Laemmli 1970) were prepared at 12 % acrylamide. First the separation gel was prepared following the Table 1 recipe, all the components were mixed and poured in to the assembled gel plates, then a fine layer of 2-propanol was added, and the gel was left to polymerize for 45-60 min at 25-30 $^{\circ}$ C. Once the gel was polymerized, the concentrator gel was added, prepared following the recipe of Table 2, then the combs were inserted between the plates. Again concentrator gel was left to polymerize 20-30 minutes at 25-30 $^{\circ}$ C.

Table 1: SDS-PAGE separator gel recipe.

Component	Volume for two gels of 0.75 mm at 12 % acrylamide.
Deionized water	3.4 mL
1.5 M Tris/HCl buffer pH 8.8	2.5 mL
SDS (20%)	50 μ L
30 % Acrylamide/ Bisacrylamide	4.0 mL
APS (100 mg/ mL)	50 μ L
TEMED	5 μ L

Table 2: SDS-PAGE concentrator gel recipe.

Component	Volume for two gels of 0.75 mm at 12 % acrylamide.
Deionized water	3.075 mL
0.5 M Tris/HCl buffer pH 6.8	1.25 mL
SDS (20%)	25 μ L
30 % Acrylamide/ Bisacrylamide	0.67 mL
APS (100 mg/ mL)	25 μ L
TEMED	10 μ L

Electrophoresis was run at 200 V for 40-45 minutes with 250 mM Tris, 1.92 M glycine, 1% SDS, pH 8.3 as running buffer.

2.3.2 Sample Denaturalization and Loading

Normal samples were denaturalized in Laemmli loading buffer 4x (10 % SDS, 40 % glycerol, 20 % 2-mercaptoethanol, 0.01 % bromphenol blue and 0.250 M Tris/HCl, pH 6.8) at 100 °C for five minutes. Once the samples were cooled, were loaded in the gel.

Urea treated samples were denaturalized with a urea solution (8 M urea, 10 mM Tris/HCl pH 10.0), at room temperature for 15 minutes before loading in the gel. The loading buffer consist in a 0.2 % solution of Bromophenol Blue, 40 % of glycerol. Samples were mixed at 50 % with this solution and loaded in the gel.

2.3.3 Colloidal Coomassie Brilliant Blue Gel Stain

Blue silver colloidal coomassie Brilliant Blue (Candiano et al. 2004) was prepared mixing 10 mL of deionized water with 12 mL of orthophosphoric acid (85 %), then 10 grams of ammonium sulfate were dissolved in this mix. When the ammonium sulfate has dissolved, 120 mg of Brilliant Blue G 250 was added and left under stirring till all the solids have dissolved. Then 48 mL of water and 20 mL of

methanol were added while stirring the mix. The solution is stable for 6 month at room temperature.

Before staining, gels were fixed for 20 minutes in ethanol/ acetic acid/ water solution (40/10/50 v/v), then washed two times with deionized water. Colloidal Coomassie was then poured over the gels and left in an orbital shaker overnight. Destaining of the gels is not necessary with this method. Gels were keep in deionized water for further analysis.

2.4 Siliceous supports preparation

Several siliceous supports were used in the Chapters 5 and 6, three methods were used to prepare silicon particles with differ sizes and properties, namely: fumed silica nanoparticles (FsNp), spherical particles (spNp) and siliceus mesocellular foam (SCM Foam). For comparison assays, commercial particles were purchased from Sigma-Aldrich: Silica Gel Spherical (40-75 μm , 75-200 μm) with 0.7-0.9 cm^3/g pore volume, and 3 aminopropyl functionalized silica gel (40-63 μm). The 3 aminopropyl functionalized silica gel was used without further modification, the others were functionalized with (3-Aminopropyl) triethoxysilane (APTES) as commented below.

2.4.1 Fumed silica nanoparticles (FsNp)

Fumed silica nanoparticles (FsNp), were prepared as reported Cabana et al (2009) five hundred milligrams of silicon dioxide amorphous were suspended in 25 mL of phosphate buffer (30 mM NaH_2PO_4 , 40 mM Na_2HPO_4), the suspension was sonicated thirty minutes in a Branson sonifier bath. The particles have a mean size of 0.007 μm but can form aggregates of several micrometers.

2.4.2 Spherical particles (SpNp)

Spherical particles (SpNp) were produced by Stöber method (Stöber et al. 1968) with slightly modifications. Briefly, five milliliters of ammonia were dissolved in 50 mL of absolute ethanol, then one milliliter of tetraethyl orthosilicate (TEOS) dissolved in one milliliter of ethanol was added dropwise while stirring rapidly. The clear solution turned murky and was left stirring overnight, the reaction scheme was resumed in Fig 1. Then the precipitates were washed and centrifuged at $4500 \times g$, trice with ethanol and trice with water, and left to dry in a furnace at $60 \text{ }^\circ\text{C}$. The particles are solid, spherical and have a mean size of $100 \text{ }\mu\text{m}$.

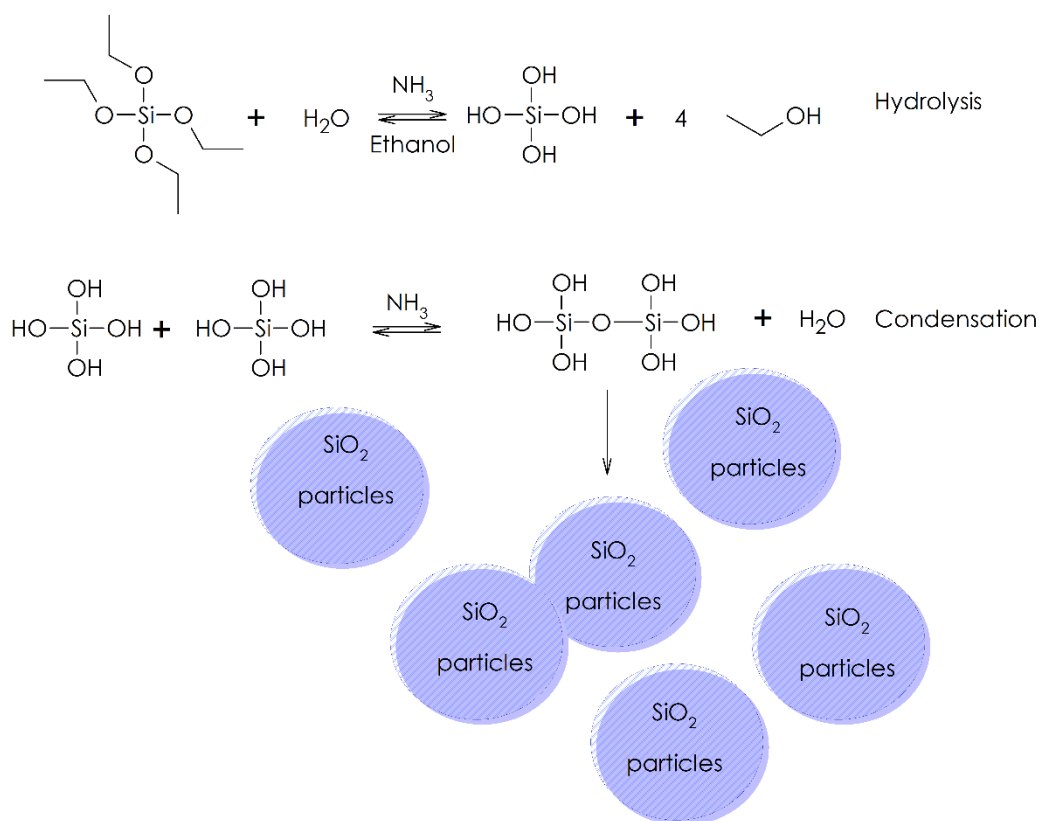


Fig 1: Stöber method (Stöber et al. 1968) for preparing silica nanoparticles.

2.4.3 Siliceous mesocellular foam (SCM Foam)

Siliceous mesocellular foam (SCM Foam) was synthesized by the literature procedure (Han et al. 2006). Four grams of Pluronic © was dissolved in 75 mL 0.016 M HCl, then 3.4 mL of 1, 3, 5 trimethylbenzene was added and the solution rapidly stirred while heating to 40 °C. After two hour 9.2 mL of TEOS was added and the mixture was further heated over 20 hours without stirring. To the solution was added 46 mg of NH_4F in five milliliters of water and the mixture was transferred to an autoclave (100 °C) for 24 hours, a briefly preparation overview was showed in Fig 2. The material was filtered, washed with ethanol and dried at 60 °C in a furnace. The particles were then calcinated at 550 °C in a muffle furnace for 8 hours.



Fig 2: SMC foam preparation.

2.4.4 Silica functionalization.

FsNp, SCM foam, and the porous silica gel purchased from Sigma Aldrich were functionalized following the method reported by Cabana et al. (2009). Briefly, five hundred milliliters of APTES were added to 500 mg of FsNp, to 100 mg of SCM foam or 100 mg of porous silica gel, suspended in 20 mL of phosphate buffer pH 7.0, and the solution was incubated for at least 12 hours. Then the particles were centrifuged (4500×g) and washed with phosphate buffer until no APTES was present in the supernatant. SpNp were dissolved in 12,5 mL of acetone with 4 % APTES and the mixture was left heating (45 °C) for 24 hours, then centrifuged and washed with water and ethanol. The functionalization of silicon particles with ethoxy silanes by condensation of precursors in mild acid pH is showed in Fig 3.

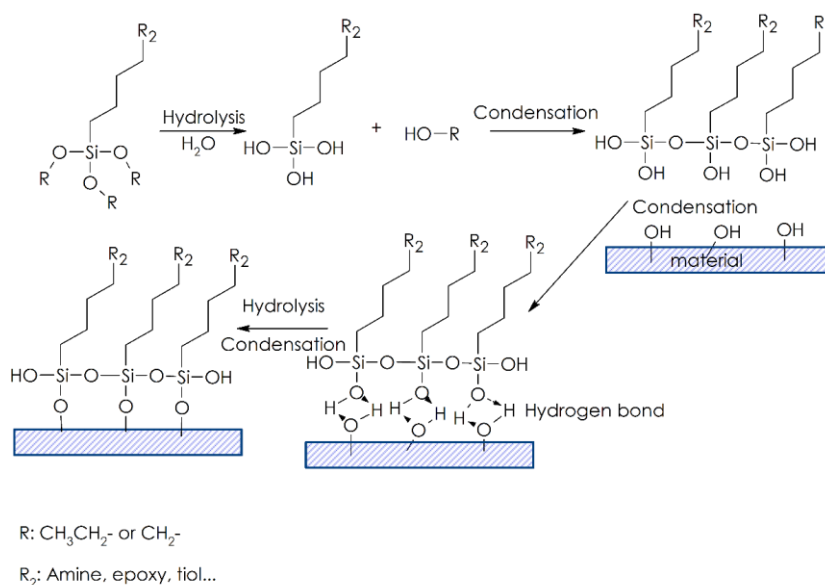


Fig 3: Silica particles modification with me/ethoxy silanes.

2.5 Fluorescence principles

Luminescence is a property of some substances to emit light when they are irradiated, it occurs from electronically excited states. Luminescence is divided in fluorescence and phosphorescence, depending on the excited state. One substance is fluorescent when excited in singlet state, the electron in the excited orbital has opposite spin to the electron in the ground-state orbital. Consequently the return to the ground state is permitted (as the electrons have contrary spins, and can be paired), it occurs rapidly with the emission of a photon. Fluorescence lifetimes (time between the excitation and the return to ground-state) are around 10 nanoseconds, while phosphorescence lifetimes are around milliseconds. Phosphorescence excited in triplet state, so the spins of the excited and ground states are parallel. This transition is forbidden, because is not possible to have two electrons with the same spin in the same orbital. The emission and return to ground-state is slow, increasing the lifetime of the luminescence. Phosphorescence usually appears in solids, however some molecules which contains iodine or bromine atoms are usually phosphorescent. Also, metal-ligand complexes displays mixed singlet and triplet states. This complexes have lifetimes of hundred nanoseconds to several milliseconds. Fig 4 shows the Jablonski diagram of fluorescence and phosphorescence. A fluorescent molecule absorbs light and excited to S_1 or S_2 energy levels, then relaxed to the lowest vibrational level S_1 (Internal conversion), emission of light occurs from this level to return to ground state S_0 . Molecules in the level S_1 can undergo a spin conversion to the first triplet state T_1 , this conversion is called intersystem crossing and produces phosphorescence (Lackowicz 1983).

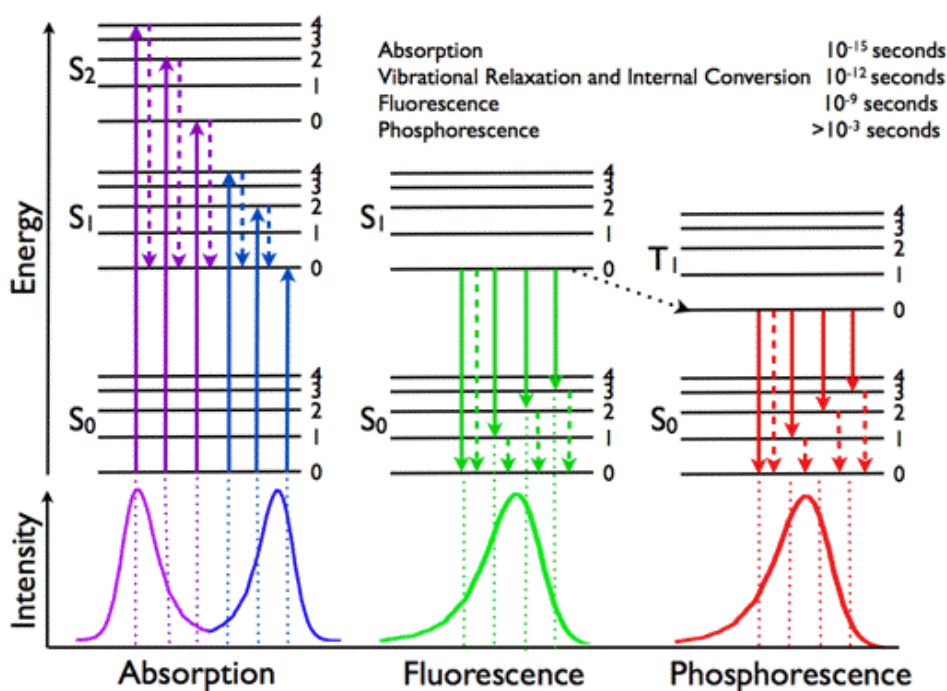


Fig 4: Jablonski diagram illustrating the fluorescent and phosphorescent phenomena.

Quinine is an aromatic molecule (present in the tonic water) which exhibits blue fluorescence (450 nm) when excited with ultraviolet light and was the first fluorophore to be described. Many other fluorophores have appeared and many others have been synthesized within the years, such as fluorescein, rhodamine, pyridine or the recent studied elements group of lanthanides (like terbium or europium).

2.5.1 Fluorescence characteristics

Fluorescent molecules share several characteristics in their fluorescent emission:

The Stokes Shift, which is defined as the distance between the maximum peak of excitation spectrum and the maximum peak of the emission spectrum.

The Kasha's rule, which reports that the emission spectra is usually independent of the excitation wavelength. Although the molecule can be excited to higher vibrational

levels, the emission of light is only produced from the lowest vibrational level to the ground state.

The mirror image rule, typically excitation and emission spectrum are specular images from each other. Exception to this rule are the pH sensitive fluorophores or the quinine.

Fluorescence lifetime: as commented before the fluorescence lifetime is the time to return from the excited state to ground state.

Quantum yield: described as the ratio between the number of photons emitted and number of photons absorbed, largest quantum yield more brightness have the fluorophore.

2.6 Principles of Fluorescent Anisotropy

Measurement of fluorescent anisotropy is a technique with multiple applications in biochemistry and medicine. Anisotropy provides information on the size and shape of a protein, protein-protein interactions (Heyduk et al. 1996), protein-molecule associations, membrane fluidity (Jähnig 1979) or immuno-assays (Lakowicz et al. 1997).

Anisotropy measurements are based on the photoselective excitation of fluorophores by polarized light. Fluorescent molecules prefer to absorb photons whose electric vectors are parallel to the transition moment of the fluorophore. In an isotropic solution fluorophores are oriented randomly, so when excited with polarized light, only the molecules oriented parallel to the electric vector of excitation will be excited (photoselection) and will emit light (Lakowicz 1999) as depicted in Fig 5.

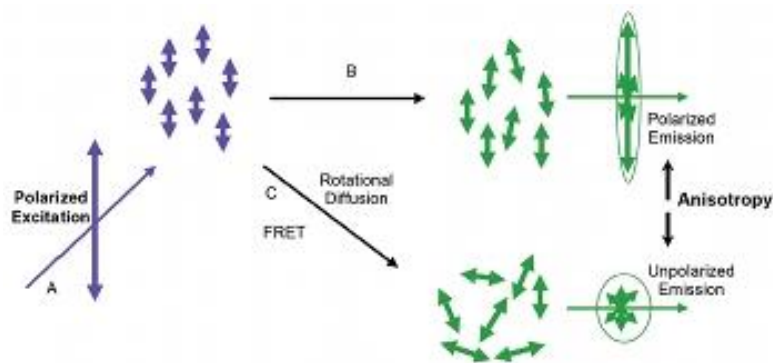


Fig 5: Photoselective excitation of the fluorophores caused by polarized light.

Anisotropy (A also denoted r in some references) is calculated with Eq. 1:

$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (\text{Eq. 1})$$

Where I_{\parallel} is the fluorescent intensity in with the polarimeters in parallel, since in the measurements the excitation polarimeter is set in vertical I_{\parallel} is also denoted I_{VV} . Likewise I_{\perp} is the fluorescent intensity in perpendicular, where the excitation polarimeter is set in vertical and the emission polarimeter in horizontal, is also denoted like I_{VH} . The anisotropy measurement diagram is depicted in Fig 6.

Anisotropy can be expressed as polarization (P) (Eq. 2)

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (\text{Eq. 2})$$

The parameters are easily converted one in other with Eq. 3. Although using anisotropy is usually preferred to polarization, because the anisotropy is normalized with the total intensity $I_T = I_{\parallel} + 2I_{\perp}$, some text still used polarization.

$$P = \frac{3A}{2+A} \quad (\text{Eq. 3})$$

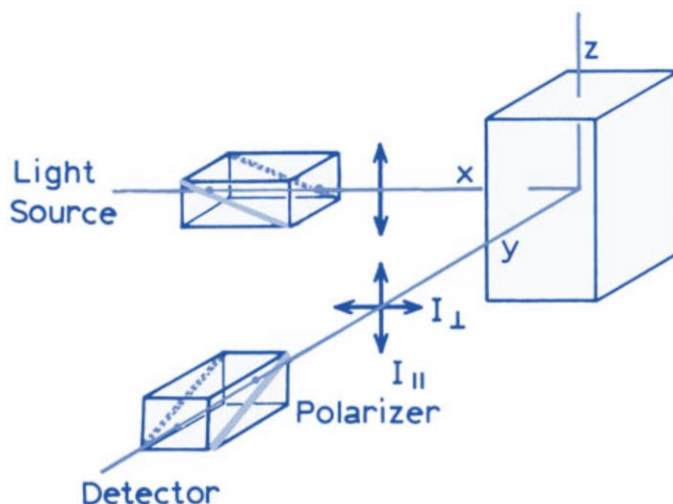


Fig 6: Anisotropy measurements outline.

Anisotropy measurements are based in Perrin equation (Eq. 4):

$$\frac{A_0}{A} = 1 + \frac{\tau}{\theta} = 1 + 6D\tau \quad \text{(Eq. 4)}$$

Where A_0 is the fundamental anisotropy, τ is the fluorescence lifetime, θ the rotational correlation time and D is the rotational diffusion coefficient. The equation can be rearranged as Eq. 5:

$$A = \frac{A_0}{1 + \frac{\tau}{\theta}} \quad \text{(Eq. 5)}$$

Using this equation is possible to calculate the anisotropy of the fluorophores in different solvents or fluorophores bond to a macromolecule. When the fluorophore is free, the correlation time is lower than the life time, the obtained anisotropy is lower than the fundamental anisotropy. When the fluorophore binds to a macromolecule the correlation time increased, because the complex is bulkier, thus the measured anisotropy for the complex will increase. Anisotropy measurements are ideal for measuring association of proteins with other macromolecules, because is linear with the correlation time. Usually the assays contains a fluorescent-labeled ligand, which binds to a non-fluorescent receptor. Being the ligand much more smaller than the receptor, its anisotropy will be low, and will increase when the ligand binds to the

receptor as the formed complex will be bulkier. Fig 7, showed two examples of anisotropy uses, a) the association/dissociation of two proteins and b) an immunoassay measurement where one antigen is labeled fluorescent and the other no, the complex antigen/antibody will have high anisotropy, but when the unlabeled antigen displaced the labeled one the anisotropy will decrease.

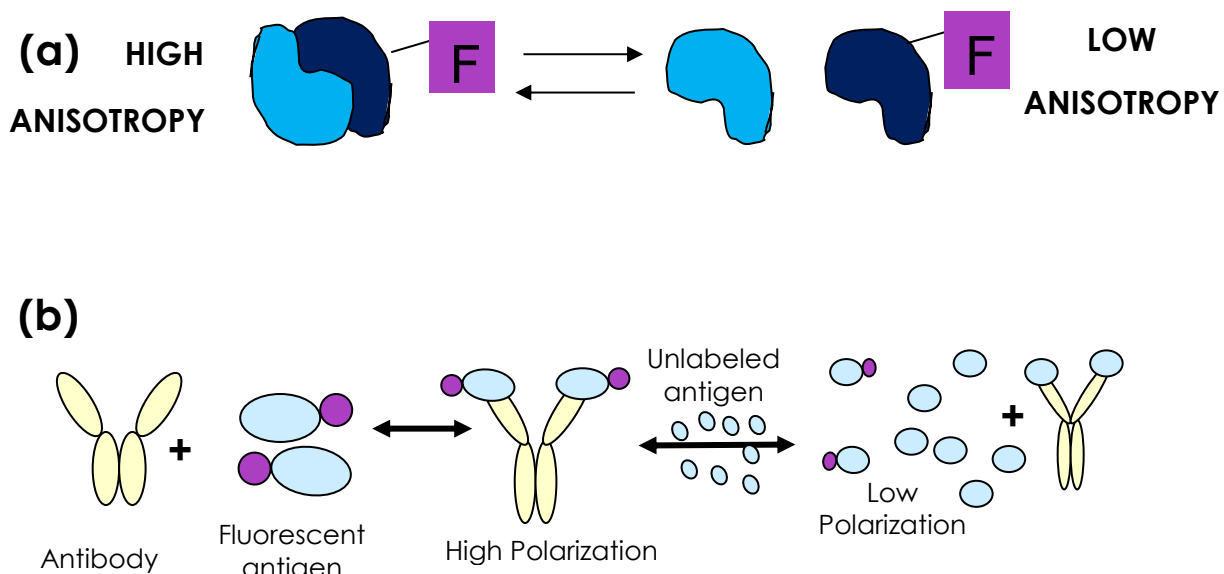


Fig 7: Anisotropy uses **(a)** protein interaction and **(b)** immunoassay

2.7 Software used

Design of experiments and ANOVA analyses were made with the Statgraphics Centurion XVI.i software. Figures and kinetic fits were made with SigmaPlot (SPW 11) software. Chemical schemes were made with ChemSketch software. Densitometry of the gel images were made with the free software imageJ.

3 References

- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1–2), pp.248–254.
- Cabana, H. et al., 2009. Immobilization of laccase from the white rot fungus *Corioloropsis polyzona* and use of the immobilized biocatalyst for the continuous elimination of endocrine disrupting chemicals. *Bioresource technology*, 100(14), pp.3447–3458.
- Candiano, G. et al., 2004. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis*, 25(9), pp.1327–1333.
- García-García, M.I. et al., 2014. First Functional and Mutational Analysis of Group 3 N-Acetylneuraminase Lyases from *Lactobacillus antri* and *Lactobacillus sakei* 23K. *PloS one*, 9(5), p.e96976.
- Han, Y., Lee, S.S. & Ying, J.Y., 2006. Pressure-driven enzyme entrapment in siliceous mesocellular foam. *Chemistry of materials*, 18(3), pp.643–649.
- Heyduk, T. et al., 1996. Fluorescence anisotropy: rapid, quantitative assay for protein-DNA and protein-protein interaction. *Methods in enzymology*, 274, pp.492–503.
- Jähnig, F., 1979. Structural order of lipids and proteins in membranes: evaluation of fluorescence anisotropy data. *Proceedings of the National Academy of Sciences*, 76(12), pp.6361–6365.
- Lackowicz, J.R., 1983. Principles of fluorescence spectroscopy. *Plenum Press*, (New York, 1983) Chapter, 5, pp.111–150.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature*, 227, pp.680–685.

Chapter 3

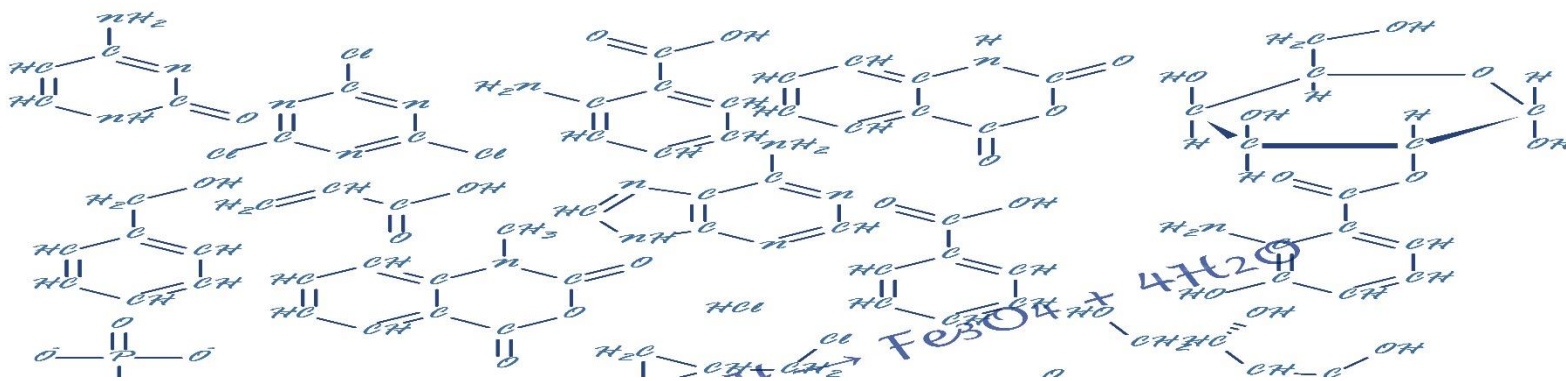
Lakowicz, J.R., 1999. Fluorescence anisotropy. In *Principles of fluorescence spectroscopy*. Springer, pp. 291–319.

Lakowicz, J.R., Szmecinski, H. & Terpetschnig, E., 1997. Long lifetime anisotropy (polarization) probes for clinical chemistry, immunoassays, affinity assays and biomedical research.

Smith, P. et al., 1985. Measurement of protein using bicinchoninic acid. *Analytical biochemistry*, 150(1), pp.76–85.

Stöber, W., Fink, A. & Bohn, E., 1968. Controlled growth of monodisperse silica spheres in the micron size range. *Journal of colloid and interface science*, 26(1), pp.62–69.

Chapter 4



Improving the activity and stability of
CLEAs and Magnetic CLEAs with
epoxides.

Abstract

Among the enzyme immobilization techniques crosslinked enzyme aggregates (CLEAs) have shown promising results in biocatalysis, because the easy preparation, versatility and low cost. The method involves the precipitation of enzymes with ammonium sulfate or an organic solvent and the posterior crosslinking with glutaraldehyde. However the Schiff base produced with glutaraldehyde is reversible and could be broken with acids or bases releasing proteins to the reaction medium, to solve this problem we propose to replace glutaraldehyde with diepoxide compounds obtaining an irreversible secondary amine bond. This change assure no leaking of protein during the biocatalytic process avoiding contamination of final products and enzyme loss. Also improves the fabrication of biocatalyst, while Schiff base is favored at mildly acid pH, epoxide reaction could be made at preferred enzyme pH, assuring its structural stability and catalytic performance.

The method have been successfully implanted in the production of aldolase crosslinked aggregates (CLEAs) and also in the production of aldolase magnetic CLEAs which include the incorporation of magnetic nanoparticles (9-12 nm) to magnetic enzyme aggregates. The obtained aldolase CLEAs retains 98 % activity yield whilst the magnetic CLEAs an 80 %.

Keywords: enzyme immobilization, crosslinking enzyme aggregates, magnetic crosslinking enzyme aggregates, epoxides, covalent bond, Schiff base, glutaraldehyde

1 Introduction

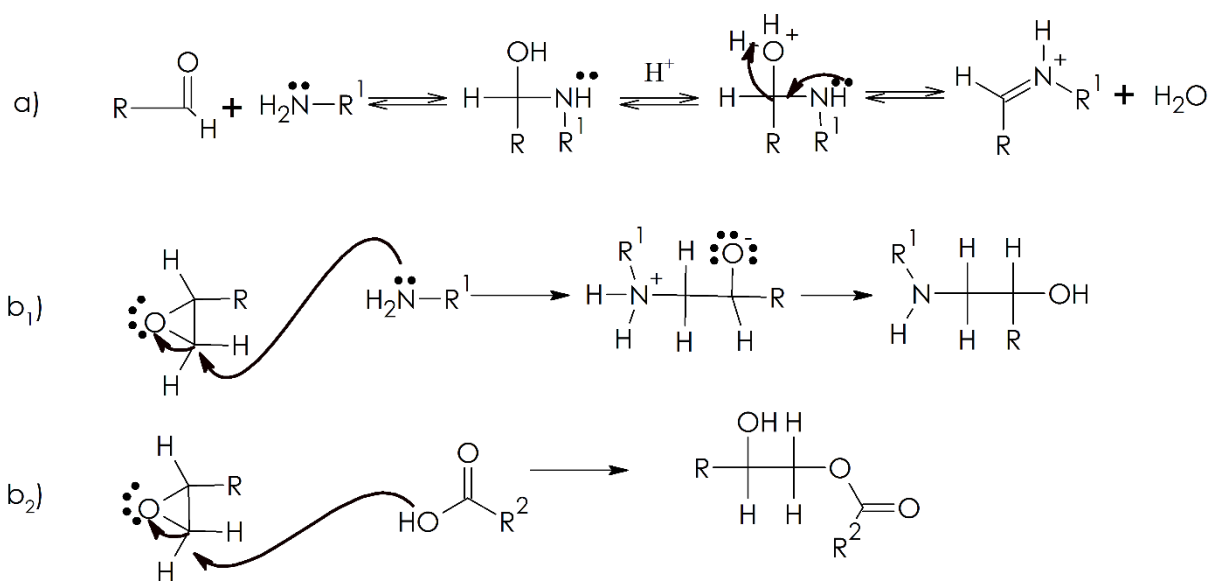
Biocatalysis is a sustainable methodology for chemical process due the mild working conditions (low pressure, moderate temperature and physiological pH) used, and great selectivity, in most of the cases there are no secondary reactions and by-products avoiding the necessity to purify the final product reducing costs.

However, industrial applications of enzymes are not widespread due to its lack of operational stability and the difficulty of separating and recovering the biocatalyst. All these problems are solved by immobilizing the enzyme (Sheldon 2007). The immobilized enzyme presents high operational stability, prevents contamination of the final product may be separated and easily recycled, also can be connected to tanks fixed or fluidized bed, for use in continuous processes.

Among the available methods of immobilization, CLEAs (cross-linked enzyme aggregates) preparation is an economically sustainable technology, because it does not need expensive supports, or a highly pure enzyme, it can be prepared from crude extracts (saves in purification costs), but sometimes present problems if the enzyme concentration is low (Sheldon 2011) also this technology uses economic chemical reagents that are commercially available in industrial quantities. The CLEAs exhibit about 100% of catalytic activity, storage and operational stability.

An improved immobilization method to prepare crosslinked enzyme aggregates (CLEAs) (Schoevaart et al. 2004; Mateo et al. 2004; Sheldon et al. 2003) is proposed, substituting the widely used crosslinked reactive glutaraldehyde by di- or tri-epoxide compounds. The reaction of enzymes with the aldehydes resulted in a Schiff base, which was stable at physiological pH but dissociated at basic pH. Usually it is necessary to reduce the Schiff with sodium borohydride to form a stable linkage. Substituting glutaraldehyde by diepoxide allows the covalent crosslinking of enzymes without the need for subsequent reduction. The reaction mechanism of the epoxy and aldehyde with amines is shown in Scheme 1. As can be seen the epoxy reaction is not

reversible and forms stable secondary amines, whilst the aldehyde reactions are reversible and form semi-stable imines, which can be broken with aqueous acids or bases (Walt & Agayn 1994; Caballero Valdés et al. 2011). Also as shown in Scheme 1 b₂, epoxides can react with carboxylic acid to form esters, this reaction is not possible with aldehydes.

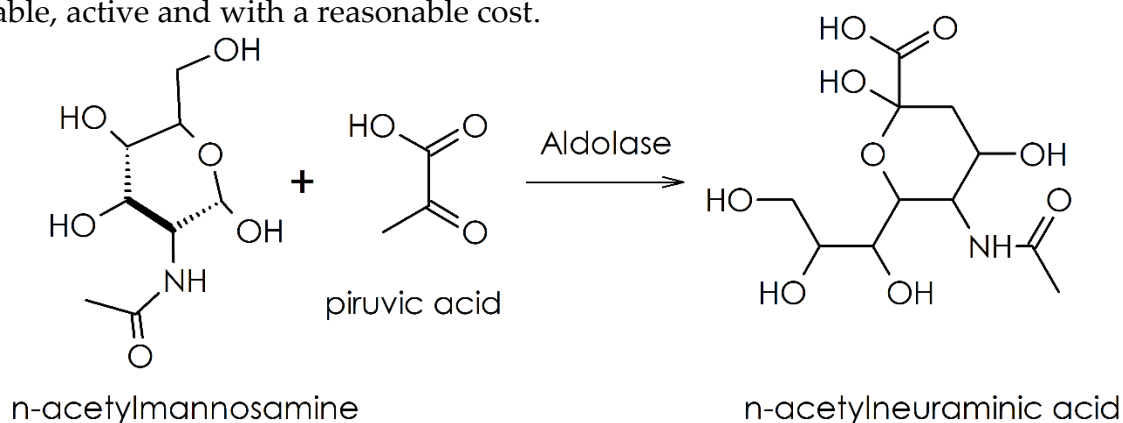


Scheme 1: Mechanism for: **(a)** aldehyde-amine reaction and Schiff base formation, **(b₁)** epoxide-amine reaction and the formation of a secondary amine and **(b₂)** epoxide-carboxylic acid reaction and ester formation.

Attempts have been made to improve CLEA production through the incorporation of natural polymers like dextran (Mateo et al. 2004) chitosan (Arsenault et al. 2011) or alginate (Fundueanu et al. 1999) or the use of synthetic polymer coated with BSA (Jasti et al. 2014), while a multitude of enzymes have been used such as peroxidase (Šulek et al. 2011), laccase (Roy & Abraham 2006), penicillin acylase (Mateo et al. 2004; Wilson et al. 2009), invertase (Talekar et al. 2010), trypsin (Jasti et al. 2014) or aldolase (García-García et al. 2011) or combination of the same in combi-CLEAs (Talekar et al. 2013). However this paper describes the first attempt to replace glutaraldehyde with a diepoxy reagent.

The carrier-free CLEA preparation protocol (Devi et al. 2009; Gupta et al. 2009; Šulek et al. 2011) was optimized to use the epoxide crosslinking compounds. The overall protocol begins by precipitating the enzymes with an organic solvent (acetone, 2-propanol) (Sheldon 2011) or with saturated ammonium sulfate (Talekar et al. 2010). Then the crosslinked reagent is added and left to react for 8-24 hours. Once the aggregates are formed, they are centrifuged and washed until no glutaraldehyde remains. The aggregates are homogenized with Fast-prep device following the method described by García-García (García-García et al. 2011). Magnetic CLEAs shows the advantage of being separated from the dissolution without centrifugation by means of a neodymium magnet.

The enzyme chosen to prepare the improved CLEAs and magnetic CLEAs is the n-acetyl-D-neuraminic acid (Neu5Ac) aldolase of *Lactobacillus antri* cloned, sobreexpressed and purified in our lab. Neu5Ac Aldolase it is a key enzyme in the biotechnological production of N-acetyl-D-neuraminic (sialic acid). The enzyme catalyzes the aldol condensation of N-acetyl-D-mannosamine and pyruvate to produce sialic acid Scheme 2, which is an intermediate for the production of antiviral zanamivir, GlaxoSmithKline: (Liese et al. 2006) Relenza®. Therefore it would be important from the industrial point of view, to obtain Neu5Ac aldolase CLEAs are stable, active and with a reasonable cost.



Scheme 2: Aldolase catalytic transformation of n-acetylmanosamine in n-acetylneuraminic acid.

2 Materials and Methods

2.1 Materials

Sodium phosphate and sodium pyruvate were supplied by Fluka. Glycerol diglycidyl ether (Technical grade), 1-4 Butanediol diglycidyl ether (> 95 % purity), Diethylene glycol diglycidyl ether (technical grade (50 % GC)), L-lactate dehydrogenase from rabbit muscle (133 U/mg) and (3-Aminopropyl) triethoxysilane (APTES) were purchased from Sigma Aldrich. NADH and ammonium sulfate were obtained from Roth and n-acetylneuraminic acid from Carbosynth. Other reagents were supplied by Sigma-Aldrich.

2.2 Aldolase production

The enzyme (Neu5Ac Aldolase) used, was cloned and purified in our laboratory as described Garcia-Garcia (García-García et al. 2014). The sequenced genome of *Lactobacillus antri* contains the nanA gen (828 bp), that belongs to a suspected n-acetylneuraminiclyase or Neu5Ac aldolase (NAL). The gen was cloned in pET 28a, and sobreexpressed in *E. coli* Rosetta 2. The clone with best activity, was cultured in TB media and induced with IPTG (Isopropyl β -D-1-thiogalactopyranoside) overnight at 30 °C. The enzyme was purified in two steps, ultrafiltration and FPLC affinity chromatography with a HisTrap column, till electrophoretic purity.

Lysines, aspartic acids, glutamic acids, isoelectric point and Grand average hydropathicity index (GRAVY) were calculated using ProtoParam tool from ExPASy Proteomic Server (<http://web.expasy.org/cgi-bin/protparam/protparam>). The computational analysis showed a molecular weight of 33372.41 Da and a theoretical isoelectric point of 5.90. The amino acid composition of the enzyme was 7.2 % Lys, 2.3 % Arg, 5.6 % Asp and 5.2 % Glu. Resulting in 29 positively charged residues and 33 negatively charged ones of 305 amino acids. GRAVY value was -0.038 indication of a hydrophilic protein.

Protein concentration was determined with Bradford reactive (Bio-Rad) (Bradford 1976) and bovine serum albumin (BSA) as standard.

2.3 CLEAs synthesis

Neu5Ac aldolase CLEAs were prepared as (Schoevaart et al. 2004; Šulek et al. 2011) at 4 °C with our modifications, in a 20 mL flask equipped with a magnetic stirred 1 mL of enzyme was added (2 mg/ mL) and 100 µL of sodium pyruvate (5 mM), as structural and active center protector, being an aldolase substrate. Then saturated ammonium sulfate (or other precipitant), was prepared in 20 mM of sodium phosphate with the pH adjusted to the desired pH (is very important to adjust the pH as the ammonium sulfate tends to be slightly acid) was added drop to drop. The volume was then adjusted to 10 mL with sodium phosphate if necessary. The enzyme was left to precipitate with mild stirring (50 rpm) for two hour in the fridge at 4 °C (normally the enzyme mixture turns a little cloudy). Then the epoxide compound was added drop to drop and the stirring speed increased (150 rpm) to favor the mix and the reaction. Passed 12 hours the formation of white/yellowish aggregates could be seen, the aggregates were centrifuged at 7000 rpm at 4 °C and washed with phosphate buffer 20 mM pH 7.0 three times, and keep in 2 mL of the same buffer.

Fast-prep treatment was made following Garcia-Garcia method (García-García et al. 2011), the aggregates were passed to a 2 mL Fast-prep suitable tubes, placed in the 24 Fast-prep (MP Biomedicals, CA, EE.UU.) and treated for 20 seconds at 4 m/s. The fast-prep treated samples was small in size and more homogeneous than the others.

Aldolase epoxy-CLEAs were prepared with different precipitants, ammonium sulfate, potassium sulfate, ethanol and acetone and different diepoxide compounds, namely glycerol diglycidyl ether (GDE), 1-4 butanediol diglycidyl ether, diethylene glycol diglycidyl ether.

Glutaraldehyde crosslinked CLEAs were prepared precipitating the enzyme (2 mg) with 76 % ammonium sulfate (pH 5.0), using 100 μ L sodium pyruvate (5 mM) as active center protector and 1 % glutaraldehyde as crosslinking reagent. After 12 hours, the aggregates were centrifuged at 7000 rpm at 4 $^{\circ}$ C and washed with 20 mM phosphate buffer pH 7.0 three times, and kept in 2 mL of the same buffer.

2.4 Mathematical optimization method

Design of experiments (DOE) and Response Surface Methodology (RSM) were used to optimize the immobilization process, the chosen mathematical model was the rotational central composite design (DRCC) of four variables at five levels. This design works the best when the goal is an optimum point that should be centered on an interest zone. DRCC is especially appropriate to take into account interactions between variables, since this experimental design required more than two levels for each variable, so that a second order approximation of the response surface can be developed. Thus, it can be identified which combination of variables generated an optimal response. The response was chosen so that the maximum enzymatic activity was obtained, Eq. 1 was used:

$$Y = \frac{CLEAsActivity}{FreeEnzymeActivity} \cdot 100 \quad (\text{Eq. 1})$$

A surface response rotational central composite design consists of a two-level factorial design, overlaid with a star design, coinciding the centers of the two designs, more no central points. In this case four variables, that be discussed below were selected, so the obtained design have 36 points (36 experiments): 16 factorial points, 10 star points and 12 identical center points, which were used to estimate the experimental error. These points formed the matrix of experiments, after carrying out the experiments, the results were used to perform a mathematical adjustment to a polynomial model, these models allowed to study the behavior of the system and find the values of the variables to maximize response, i.e. finding the optimum.

The experimental data were adjusted and analyzed by Essential regression, an Excel free macro and the Statgraphic Centurion program. The optimum was obtained by macro Excel Solver to find the maximum and minimum of a function in an interval.

2.5 Tomography microscopy of epoxy-CLEAs

Droplets of CLEAs (glutaraldehyde crosslinked) and epoxy-CLEAs were placed on a filter paper and serially dehydrated using different acetone solutions (30 %, 50 %, 70 %, 90 % and 100 %) and dried at critical point with CO₂. After gold sputtering, samples were visualized in a tomography microscope operating at 10 kV.

2.6 Stability assay

To assess the time-stability of CLEAs and Epoxy-CLEAs in comparison with the free enzyme, samples were incubated in 20 mM sodium phosphate pH 7.0 at 37 °C for several hours. At different time intervals aliquots were withdrawn and the remained activity of the samples was measured.

2.7 Magnetic CLEAs synthesis

Magnetic CLEAs were prepared following the method of Talekar (Talekar et al. 2012), with modifications. The magnetic particles were prepared by coprecipitation method (Kim et al. 2001), 13.51 g of FeCl₃ 6H₂O were dissolved in 25 mL of distilled water, 6.95 g of FeSO₄ 7H₂O were dissolved in 25 mL of 0.5 M HCl. The solutions were mixed and dropped on 150 mL of NaOH 5 M, with high stirring (1500-2000 rpm). A black precipitated with magnetic properties was immediately formed. Once all the iron solution is dropped, the mixture was neutralized (pH 7.0) with HCl 5 M. Using a neodymium magnet the supernatant was removed and the particles were washed three times with 100 mL of deionized water. The synthesized magnetic particles had a mean size of 9-12 nm.

Magnetic particles were functionalized with (3-Aminopropyl)triethoxysilane (APTES), 1 g of magnetic particles were added to 50 mL of 50 % ethanol solution under stirring, the solution was adjusted to pH 5.0 with acetic acid and NaOH, then 1 mL of APTES were added and the solution was left under stirring (150 rpm) for one hour. The particles were attracted to the flask bottom with a neodymium magnet and the supernatant was decanted, amino functionalized particles were washed once with acetone and twice with water.

Magnetic epoxy-CLEAs were prepared in a 20 mL glass flask equipped with a magnetic stirred, 1 mL of enzyme (2 mg/mL), 100 μ L of sodium pyruvate and 100 mg of particles were added under stirring (150 rpm), then 9 mL precipitant (saturated ammonium sulfate or 50 % polyethyleneglycol ₆₀₀₀ solution) with the pH adjusted were added and left at 4°C for two hours, then 100 μ L of 1-4 butanediol diglycidyl ether were added drop to drop and left to react overnight. Magnetic CLEAs were washed trice with sodium phosphate (20 mM, pH 7.0).

Bradford reactive (Biorad) (Bradford 1976) was used to quantify the remained proteins in the supernatants.

2.8 Aldolase activity measurements

Enzyme activity was determined spectrophotometrically with a coupling reaction, measuring the decrease of absorbance at 340 nm corresponding the NADH oxidation to NAD by L-lactate dehydrogenase. The aldolase produces pyruvate from the Neu5Ac cleavage, then the pyruvate is transformed to lactate by L-lactate dehydrogenase oxidizing NADH to NAD (García-García et al. 2014). All the measurements were made in sodium phosphate 20 mM pH 7.0 and 37 °C.

3 Results and Discussion

3.1 Preliminary assays

After conducting preliminary tests with precipitants of different nature and different epoxy cross-linking, results were summarized in Table 1. Ammonium sulfate was chosen as precipitant to optimize the immobilization process as was the only precipitant that did not inhibit the enzyme and DGE as crosslinking reagent. DGE was chosen because is sensibly more economic that 1-4 butanediol diglycidyl ether. However the three tested reagents produced stable insoluble CLEAs that maintain the activity implying that diepoxy reagents are good replacements of glutaraldehyde.

Table 1: Aldolase epoxy-CLEAs preparation.

Precipitant	% Precipitant	Cross-linked	Cross-linked (μL)	pH	Fast-Prep	Cross-Linked Time (h)	CLEA activity
(NH₄)₂SO₄ Saturated	90		0	7.0			0
	70		100	7.0	No	24	15.01
	60	Glycerol diglycidyl ether (GDE)	200	7.0	Yes	48	53.42
	50		200	7.0	No	48	14.64
	60		200	6.5	Yes	24	11.93
	60		400	6.5	Yes	24	50.70
	60	1-4 Butanediol diglycidyl ether	100	6.5	Yes	24	22.70
	60		150	6.5	Yes	24	31.19
	60		200	6.5	Yes	24	76.09
	60	Diethylen glycol diglycidyl ether	100	6.5	Yes	24	24.16
60	200		6.5	Yes	24	50.25	
K₂SO₄ Saturated	90		100	6.27	Yes	24	0
	80	GDE	100	6.27	Yes	24	0
	70		100	6.27	Yes	24	0
Acetone	90		0	7.0			0
	90		100	7.0	Yes	24	0
	80	GDE	100	7.0	Yes	24	0
	70		100	7.0	Yes	24	0
	90		0	7.0			0
Ethanol	90		100	7.0	Yes	24	0
	80	GDE	100	7.0	Yes	24	0
	70		100	7.0	yes	24	0

3.2 Mathematical optimization

From the results obtained in section 3.1, the most influential variables in the process were identified, therefore the most indicated to optimize the process. The selected variables were shown in Table 2, which also includes the ranges in which these parameters will vary in optimization.

Table 2: Chosen parameters for the optimization

Variables	Units	Levels				
		-2	-1	0	1	2
X ₁ Ammonium sulfate	mL	2	3.75	5.5	7.25	9
X ₂ Cross-linking reagent	μL	100	200	300	400	500
X ₃ pH	Adimensional	5	5.75	6.5	7.25	8
X ₄ Cross-linking time	hours	7	14.75	22.5	30.25	38

As could be seen in the Table 2 the variables will be standardized, so they are dimensionless, have the same mean and the same standard deviation.

In Table 3 the central rotational design was built with the natural values of the variables and the results obtained for the response, along with the theoretical results obtained by adjusting the data to the obtained mathematical model.

Table 3: Experimental matrix with the obtained and the predicted results for the model.

Run	Variable in CLEAs optimization				Response Y	
	X ₁	X ₂	X ₃	X ₄	Experimental Y	Estimated Y
1	3.75	200	5.75	14.75	9.65	15.21
2	7.25	200	5.75	14.75	38.57	31.67
3	3.75	400	5.75	14.75	5.08	15.88
4	7.25	400	5.75	14.75	92.94	76.42
5	3.75	200	7.25	14.75	13.33	16.02
6	7.25	200	7.25	14.75	12.73	7.31
7	3.75	400	7.25	14.75	15.87	24.93
8	7.25	400	7.25	14.75	57.89	60.28
9	3.75	200	5.75	30.25	9.60	17.60
10	7.25	200	5.75	30.25	51.02	37.40
11	3.75	400	5.75	30.25	26.39	27.25
12	7.25	400	5.75	30.25	83.43	91.12
13	3.75	200	7.25	30.25	11.42	23.38
14	7.25	200	7.25	30.25	18.41	17.99
15	3.75	400	7.25	30.25	23.98	41.26
16	7.25	400	7.25	30.25	90.07	79.95
17	9.0	300	6.5	22.5	8.28	32.64
18	2.0	300	6.5	22.5	7.68	22.51
19	5.5	500	6.5	22.5	77.25	69.44
20	5.5	100	6.5	22.5	4.83	6.81
21	5.5	300	8.0	22.5	83.19	72.39
22	5.5	300	5.0	22.5	77.78	82.75
23	5.5	300	6.5	38.0	44.11	36.19
24	5.5	300	6.5	7.0	12.05	14.13
25	5.5	300	6.5	22.5	51.97	52.08
26	5.5	300	6.5	22.5	50.83	52.08
27	5.5	300	6.5	22.5	49.89	52.08
28	5.5	300	6.5	22.5	47.23	52.08
29	5.5	300	6.5	22.5	47.61	52.08
30	5.5	300	6.5	22.5	50.20	52.08
31	5.5	300	6.5	22.5	53.48	52.08
32	5.5	300	6.5	22.5	51.11	52.08
33	5.5	300	6.5	22.5	55.85	52.08
34	5.5	300	6.5	22.5	52.13	52.08
35	5.5	300	6.5	22.5	57.39	52.08
36	5.5	300	6.5	22.5	57.31	52.08

Once the experiments were made, the results were adjusted to a second order polynomial model with the following equation (Eq. 2):

$$\text{Yield} = 52.08 + 13.78X_1 + 15.65X_2 - 2.58X_3 + 5.51X_4 - 11.75X_1^2 + 11.01X_1X_2 - 6.29X_1X_3 + 0.83X_1X_4 - 3.48X_2^2 + 2.05X_2X_3 + 2.24X_2X_4 + 6.37X_3^2 + 1.24X_3X_4 - 6.73X_4^2 \quad \text{(Eq. 2)}$$

Then the model was statistically analyzed, the analysis of significance of the regression model determines whether there is a relationship between the response variable Y and a set of regression variables X_1, X_2, \dots, X_k . To do this, statisticians tend to define the "null hypothesis". The worst case scenario is assumed saying: "The null hypothesis (H_0) is true if there is no relationship between any of the independent variables".

If the null hypothesis (H_0) is rejected, then at least one of the variables in the regression model contributes significantly to the model and can be concluded that there is a functional relationship between the response and at least one of the variables.

The analysis for the significance of the regression model is thus performed as a method of analysis of variance or *ANOVA*.

This analysis was used to summarize the evidence of the significance of the second order polynomial model obtained. The analysis of variance table (Table 4) shows the amount of variation in response due the variables (regression) and the amount of variation that is unexplained (residual or error). Due to the existence in the matrix of experimental repeated points, the unexplained variation is presented divided into pure error variation and the variation due to the inadequacy of the model or lack of fit.

Table 4: ANOVA table for the proposed model

ANOVA						
Source	SS	SS%	MS	F	F Signif	df
Regression	14.46	67	1.033	2.989	0.01157	14
Residual	7.257	33	0.346			21
LOF error	4.663	21 (64)	0.466	1.9775	0.139	10
Pure error	2.594	12 (36)	0.236			11
Total	21.72	100				35

As can be seen in the Table 4 the probability that the model is not significant is greater than the significance level alpha ($\alpha = 0.1$), therefore we accept that at least one of the terms of the model influences the response and we reject the null hypothesis. So our model is significant and can be used to describe the CLEAs production process.

The program Solver allowed us to obtain a maximum point for the response (Y) within the working interval introducing the proposed and statistically significant polynomial model. The mathematical optimization of the method for GDE as precipitant results in a 98 % activity yield, the conditions used were 76.3 % ammonium sulfate saturated at pH 5.0, 273 μ L of GDE and 23 hours of reaction. Then washed as described before and treated with fast prep 20 seconds at 4 m/s.

The analysis of the partial effects of a variable allows to establish the influence of that variable in the response. The equations of partial effects are obtained by replacing all but one variable, by its optimal coded value, thus an equation in which the response depends only on one variable is obtained.

Chapter 4

The partial effects graphic (Fig 1) showed that the variable X_1 (volume of ammonium sulfate) was the most influential in the model because it was the variable whose rate of change in relation to the response was almost exponential. However when X_1 takes values above 1 (7.25 mL), the response begins to descend.

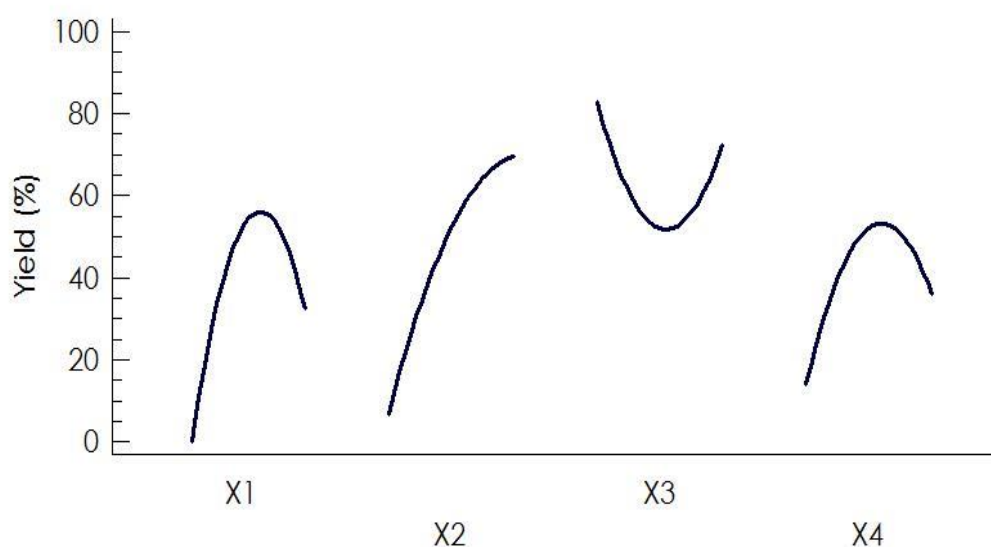


Fig 1: Partial effects graphical representation for the mathematical model.

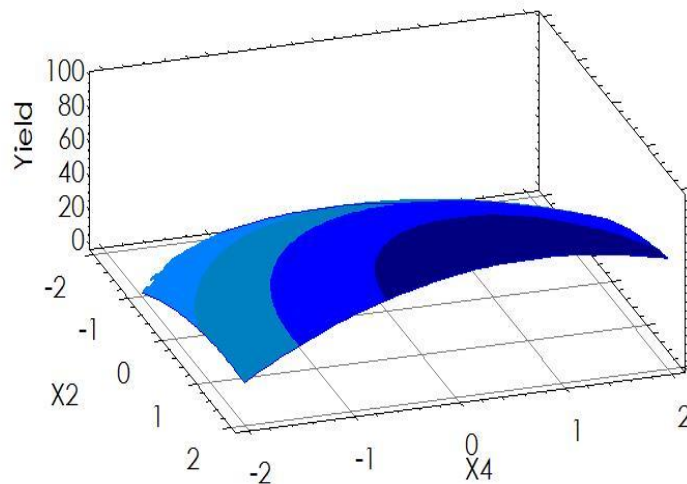
The variables X_2 and X_4 (reagent volume of cross-linking reagent and time cross-linking), were less influential in the response, however the influence was of order two, that was, the term of the equation which produces more variation in response was quadratic. It can be seen in the equation that the coefficients accompanying the quadratic terms (X_2^2 , X_4^2) are 10 times larger than the linear terms (X_2 , X_4). In the case of X_2 , the epoxy-CLEAs activity was almost lineal with the amount of epoxide added, however the activity decreased when using higher amounts of reagent, a possible indication of active centers blockage. Is the same for the reaction time (X_4), in short times little reaction between the enzyme and the cross-linked occurs, and very long times the cross-linked begins to block the active centers and activity was lost.

Chapter 4

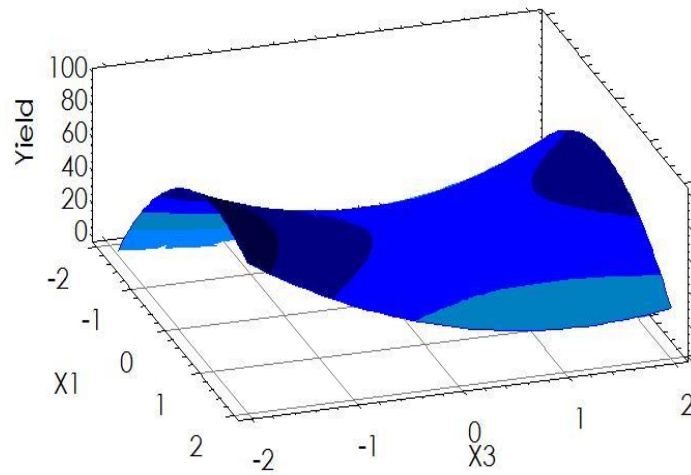
The effect of pH (X_3) was different to that observed for the other variables, since the influence of pH was inversely proportional to the response. It can be seen an increase in the response when the pH was lowered. In this case the linear effect was more pronounced, although the quadratic terms were of the same order.

A surface response is a three dimensional, or shaped contour graph of a response to two of the variables that influence it. Thus, the response is plotted on the vertical axis, while the horizontal axes variables with their coded values are represented. These graphs show the influence of each variable in the process and its interaction with other variables. Since only representations in three dimensions could be made, this limit to two the number of variables that are studied each time. All other variables remain constant at their optimum values.

(a)



(b)



(c)

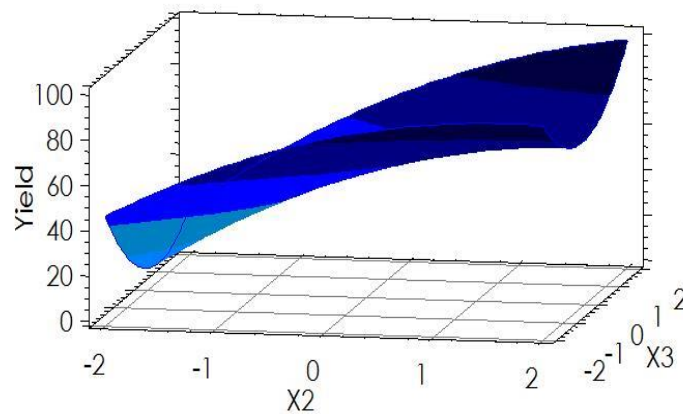


Fig 2: Surfaces response for the variables **(a)** X_2 and X_4 **(b)** X_1 and X_3 **(c)** X_2 and X_3 .

Surfaces responses were obtained for all combinations of all variables for the proposed mathematical model. However only the most representative of the process were shown in Fig 2. Of all response surfaces obtained, the most striking are those in which the pH is represented (Fig 2 (b) and (c)) in one of its axes. It can be seen that they had a characteristic "saddle" shape, i.e. the graph had a minimum around neutral pH, and both in acidic and in alkaline conditions the response was increased. This can be described as a relationship between the reactivity of epoxides and pH, the neutral pH was less favorable for crosslinking than more acidic or basic pH. Normally epoxides react with amino groups of side chains (normal reaction), the cross-linking involves the reaction of the amino groups of the lysine residues with the epoxide groups of the diglycidyl glycerol ether molecules, resulting in the formation of secondary amines (Zeeman et al. 1998). However, as already reported (Shechter & Wynstra 1956; Blank et al. 2002) the epoxy groups are capable of forming bonds with the carboxyl groups sidechain in different conditions to produce ester bonds. The reactivity towards amines and carboxyl groups supposes an advantage of the epoxides over other crosslinked that only reacts with amines as glutaraldehyde. The rate of immobilization of proteins that lacks of amine residues with glutaraldehyde is low, compelling to chemically modify the protein before cross-linking it (Rodrigues et al. 2011) with epoxides this problem is avoided as can directly react with carboxyl groups.

3.3 Validation of the model

The validation of the mathematical model aims to demonstrate that the obtained equation correctly fits and describes the response. The validation of any mathematical model must be made using different experimental results which were used for its construction, i.e. new experiments are performed and the results obtained are compared with the values predicted by the model for the conditions imposed (Mizubuti et al. 2000). To predict the theoretical values, we used an Essential Regression tool that provides a response value, with a confidence interval of 90 %, to

introduce the values of the variables in coded form. For model validation were carried out 8 new experiments, one of them in optimum conditions and the rest to get different responses, in order to compare them with the estimated responses provided by the model. The values predicted by the proposed models have been plotted against the values obtained experimentally in Fig 3. It can be seen that the fit is good, since regression coefficient is very close to the unit (0.9815).

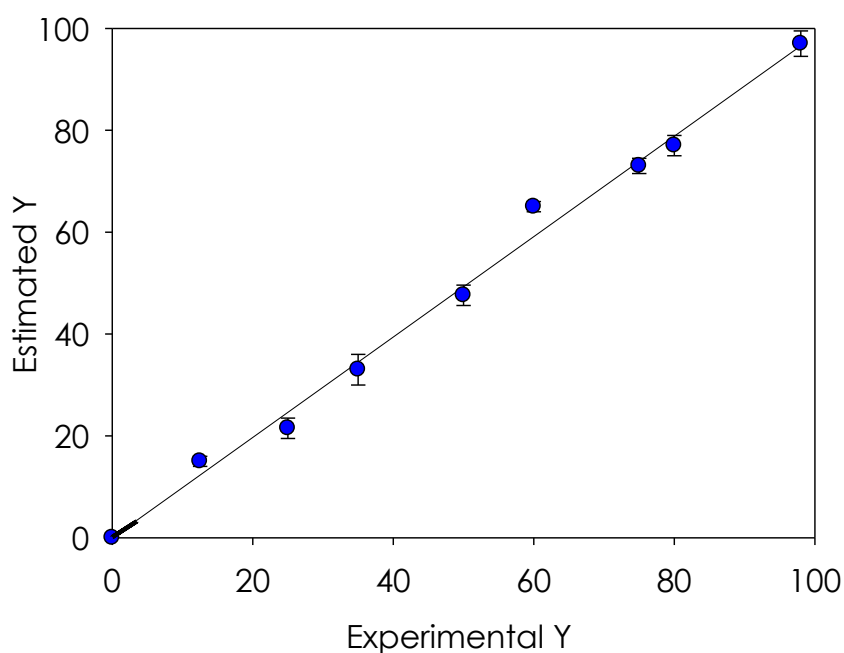
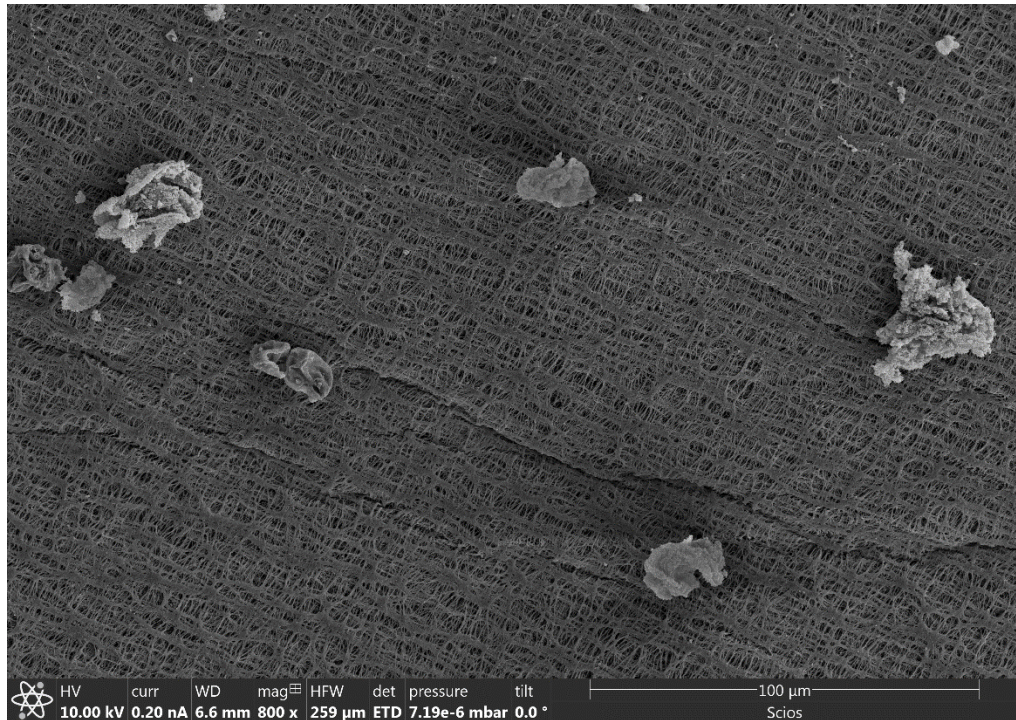


Fig 3: Validation plot (estimated Y vs experimental Y) for the mathematical model.

3.4 Scanning Electron microscopy of epoxy-CLEAs

Epoxy-CLEAs were smaller in size than glutaraldehyde-CLEAs as showed the microscope images (Fig 4 and Fig 5). Glutaraldehyde-CLEAs had a size around 25 μm , while epoxy-CLEAs had a size over 3 μm .

(a)



(b)

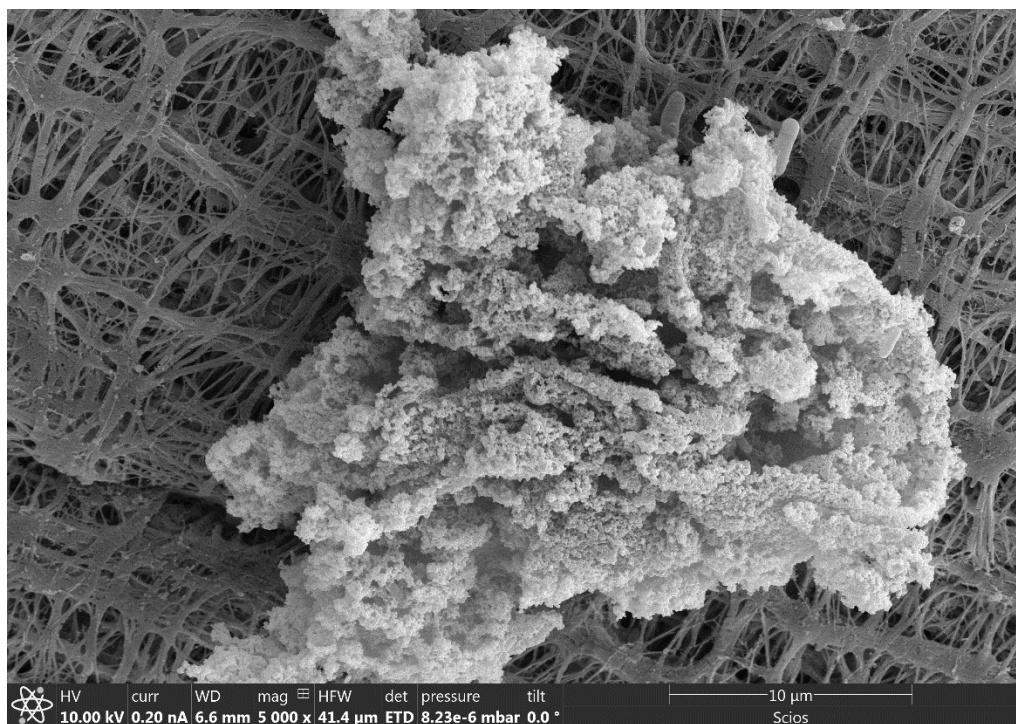
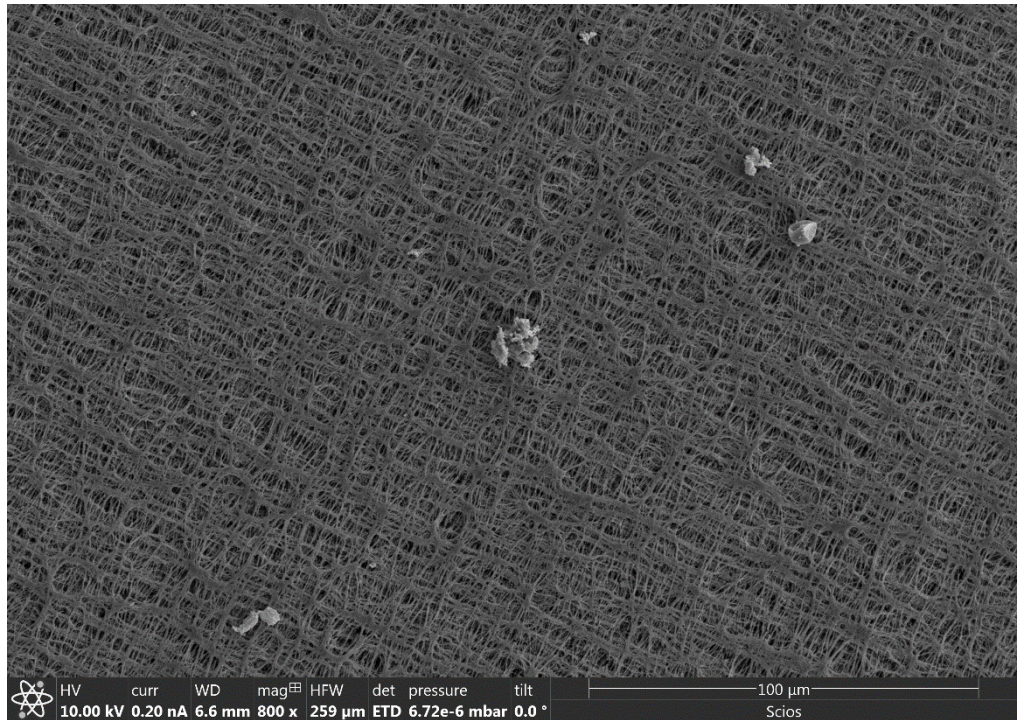


Fig 4: Glutaraldehyde-CLEAs tomography images at **(a)** 800x and **(b)** 5000x

(a)



(b)

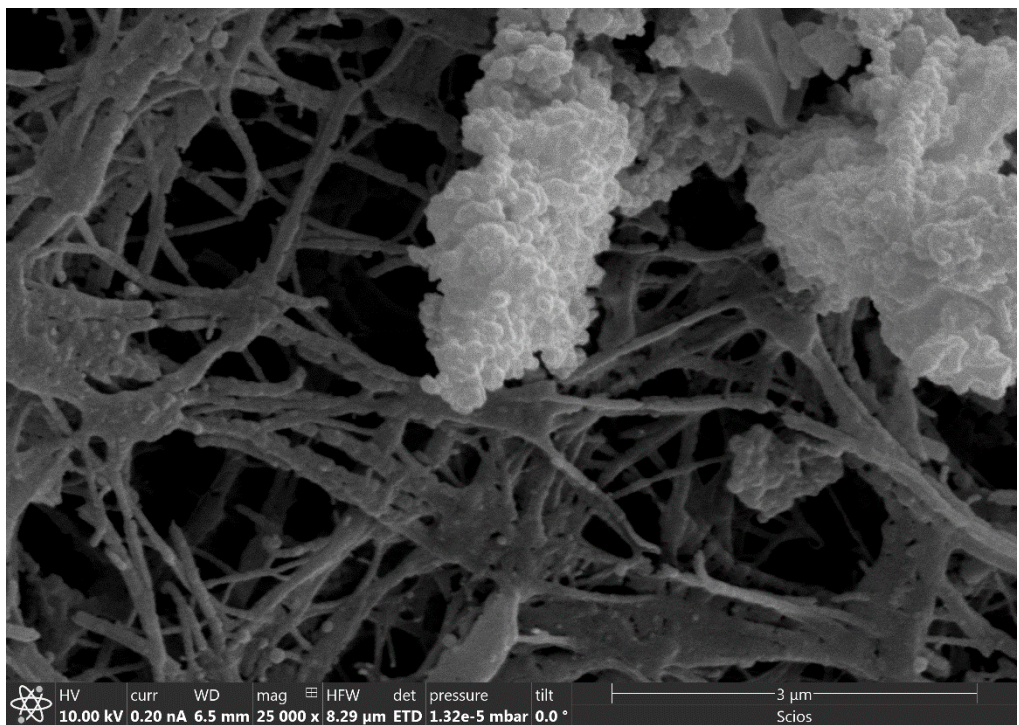


Fig 5: Epoxy-CLEAs tomography images at **(a)** 800x and **(b)** 25000x

3.5 Stability assay

The stability assay depicted in Fig 6 showed that the epoxy-CLEAs were more stable than the free enzyme and much more stable than the glutaraldehyde CLEAs. In five hours the glutaraldehyde CLEAs had lost the 50 % of its activity and the free enzyme around a 30 % while the epoxy-CLEAs maintain the 98 % of its activity.

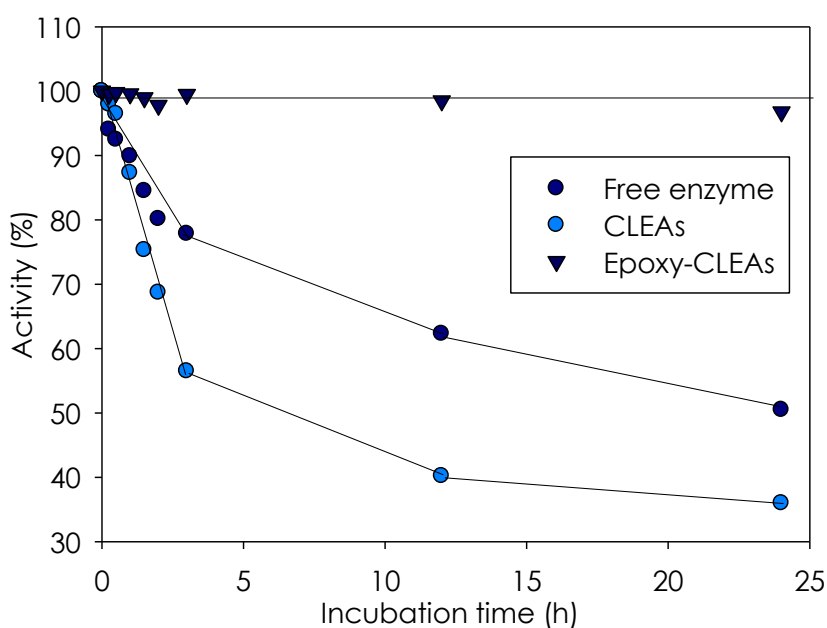


Fig 6: Stability assay for free enzyme, glutaraldehyde CLEAs and epoxy-CLEAs in 20 mM phosphate buffer pH 7.0 at 37 °C.

3.6 Magnetic epoxy-CLEAs

The good results obtained with the epoxy crosslinked enzymes encourages us to make a step forward and implemented the method for magnetic CLEAs. Magnetic CLEAs have the advantage over the CLEAs of being directly removed from the reaction medium with a magnet avoiding centrifugation steps, as demonstrated Fig 7. The procedure was the same as the CLEAs but including amino derivatized magnetic particles to crosslink with the protein, resulting in protein and magnetic particles aggregates. The precipitants selected for these assays were ammonium sulfate as it

works for the normal CLEAs and a 50 % PEG₆₀₀₀ (polyethylene glycol with an average molecular weight of 6000) solution. PEGs are well known protein precipitants, they adsorb water dehydrating the proteins and precipitating them (Atha & Ingham 1981). Several assays were carry on to asset the suitability of the method. Table 5 summarized the characteristics of the magnetic epoxy-CLEAs in comparison with the magnetic glutaraldehyde CLEAs and the normal epoxy CLEAs prepared as control. As the Table 1 showed epoxy-CLEAs and magnetic epoxy-CLEAS had better activity yield than glutaraldehyde, also with the epoxy reagent there was no enzyme present in the supernatant indicating a precipitation and immobilization complete of the enzyme added.



Fig 7: Images of the magnetic epoxy-CLEAs remove from the reaction medium with a neodymium magnet.

Table 5: Comparison of biocatalyst immobilization mass and activity yield

Aldolase biocatalyst	Enzyme immobilized (mg) ¹		Activity yield (%)	
	90% Ammonium sulfate pH 5.0	50 % PEG ₆₀₀₀ pH 5.0	90% Ammonium sulfate	50 % PEG ₆₀₀₀
Magnetic epoxy-CLEAS	2 mg	2 mg	60 %	80 %
Magnetic glutaraldehyde CLEAs	1.5 mg	1.2 mg	69 %	57 %
Epoxy-CLEAs	2 mg	2 mg	76 %	60 %

¹ Measured with Bradford reagent

The relationship between the mass of enzyme attached to the support and the precipitant pH could be seen in Fig 8, as expected the best pH for the reaction was mild acidic pH as obtained for the CLEAs, and the best precipitant was PEG 6000. The PEG 6000 dehydrated the enzymes and, consequently, precipitates them on the magnetic particles surface. When the crosslinking reagent was added the crosslinking reaction occurred on the surface of the particles, as the proteins were precipitated on the surface.

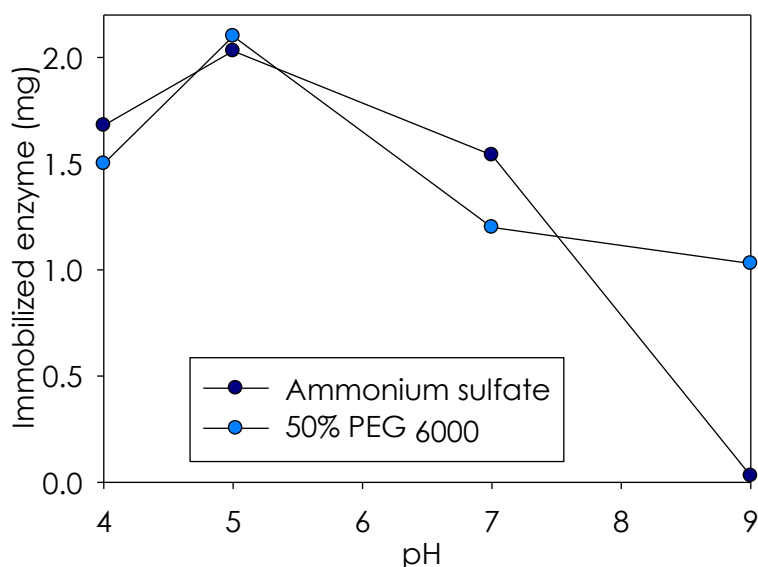


Fig 8: Relation between the immobilized enzyme and the precipitant pH, for the precipitants ammonium sulfate (●) and polyethylene glycol 6000 50 % solution (●).

The quantity of enzyme that can be attached in the magnetic support is 2 mg per 100 mg of support. Also the pH and precipitant that immobilized more enzyme are the ones with best activity recovery as shows Fig 9, reaching an 80 % activity yield in comparison with the activity yield of the same weight of free enzyme.

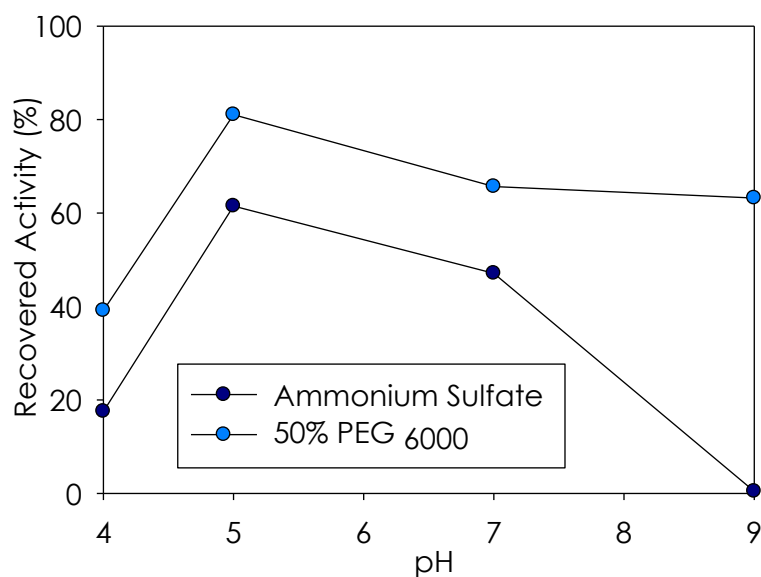


Fig 9: Relation between the activity of the immobilized enzyme and the precipitant pH, for the precipitants ammonium sulfate (●) and polyethylene glycol ₆₀₀₀ 50 % solution (●).

The immobilization rate of the precipitant at pH 5.0, measured as disappearance of activity in the supernatant was shown in Fig 10, PEG ₆₀₀₀ precipitates the enzyme faster than ammonium sulfate, in 40 minutes PEG ₆₀₀₀ shows no remaining activity in the supernatant whilst ammonium sulfate need 60 minutes to precipitate the enzyme.

The obtained results displayed the eligibility of diepoxides as crosslinking reagents, being stable reagents, ready reactive and economically sustainable.

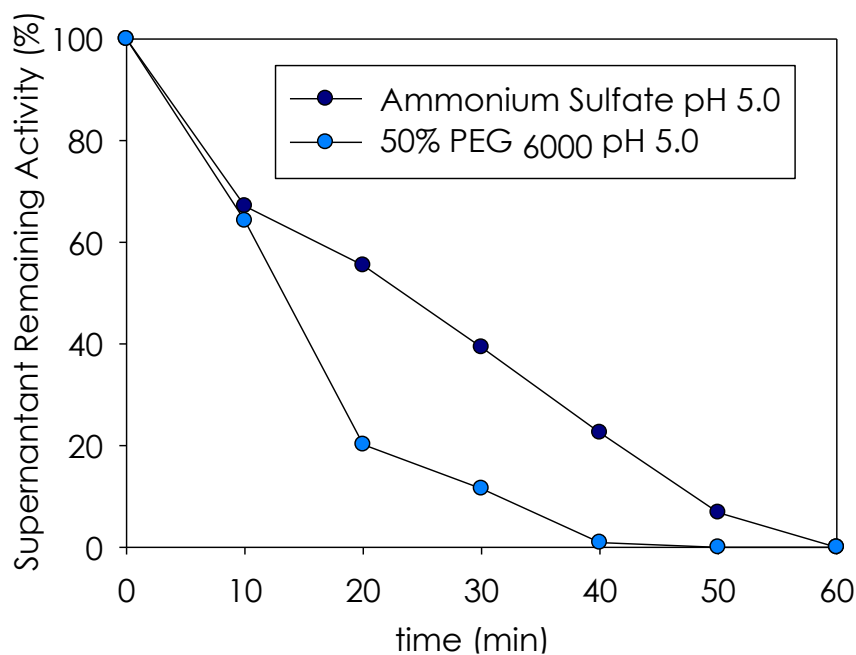


Fig 10: Immobilization rate, measured as remaining activity in the supernatant for the precipitants ammonium sulfate (●) and polyethylene glycol 6000 50 % solution (●) at pH 5.0.

In comparison with the magnetic CLEAs patented method (Sheldon et al. 2012) our magnetic epoxy-CLEAs are composed of 2 mg of enzyme per 10 mg of magnetic support as no enzyme activity was left in the supernatant, the activity yield was around 80 %, while the patented method yields a 41 % activity and immobilized around 10 mg of lipase in 100 mg of magnetic particles.

4 Conclusions

Glutaraldehyde is the most popular crosslinking reagent used in crosslinking enzyme aggregates (CLEAs) production, however it has some handle problems as its low storage stability, low reactivity with certain enzymes and the formation of reversible Schiff bases. We proposed an alternative to immobilization via cross-linked with glutaraldehyde; immobilization via cross-linked with epoxides, were tested three different epoxy reagents and produced stable and catalytically active epoxy-CLEAs.

The production of Neu5Ac aldolase (a key enzyme in the fabrication of the antiviral zanamivir; GlaxoSmithKline) epoxy-CLEAs was optimized using a mathematical approach: design of experiments (DOE) and response surface methodology. With the mathematical optimization epoxy-CLEAs with a 98 % activity yield, in comparison glutaraldehyde CLEAs only retained a 79 % of activity. Also epoxy-CLEAs were more stable than glutaraldehyde CLEAs, while CLEAs had lost the 70 % of activity in 24 hours epoxy-CLEAs loss of activity was almost negligible.

Using design of experiments (DOE) and the Methodology Response Surface (MRS), have proposed a model to describe the phenomena occurring in the preparation of CLEAs, the model has been subjected to an analysis of variance (ANOVA) to check them statistically significance. The variance analysis has shown that the proposed model has a variation of more than 65 %.

With the optimization of mathematical model the conditions under which the CLEAs reaches its peak activity have been obtained, minimizing the amount of cross-linked reagent. The resulting epoxy-CLEAs maintain all its activity. Noted that experiments had been conducted with a very low enzyme concentration (2 mg / mL), conditions not very favorable for precipitation.

The technique of the partial effects allowed to see the influence of each variable separately in the system, so it has been deduced that the activity of the CLEAs is closely

related to the amount of precipitating reagent added, it has been seen that the increase in response to increase the volume of precipitant is almost exponentially. Regarding the pH was found to have an inverse effect on the answer, that this effect is practically linear, and that the best answers are given at acidic pH, which fits perfectly with the optimal conditions of the free enzyme. It has been seen that influences the volume of cross-linked and time are small, but are mainly due to the terms of order two.

With the methodology surface response have been seen existing intrinsic relationships between process variables, it has been deduced that the most important relationship is that of crosslinking reagent with the precipitant, this relationship will provide the amount of enzyme available to react with the reagent cross-linked, are also important the ratio of the precipitant with the pH and the ratio of pH with the epoxide, these two relationships can be deduced that the epoxides are capable of reacting in basic medium with NH_2 groups of the amino acids and acid medium can react with carboxyl groups.

The duality of being capable of reacting with different functional groups, depending on the medium pH, makes epoxides good candidates to replace glutaraldehyde, as there is no need of posterior reduction with borohydride to form stable bonds and can be used in a longer pH range than glutaraldehyde. Therefore, epoxides double the possibilities of producing active CLEAs, reduces the fabrication steps and avoids handling borohydride.

Finally, the methodology is tested for magnetic CLEAs. The incorporation of magnetic particles, improves the possibilities of use these enzymatic preparations in industrial processes as ease management of the catalyst. The possibility of removing the catalyst from the reaction medium easily with a magnet facilitates its removal and reuse, prevents contamination of the final products and avoids costly centrifugation steps. The magnetic epoxy-CLEAs produces retained a 80 % of activity yield, showing the versatility of the proposed crosslinking reagents.

5 References

Arsenault, A., Cabana, H. & Jones, J.P., 2011. Laccase-based CLEAs: chitosan as a novel cross-linking agent. *Enzyme research*, 2011.

Atha, D.H. & Ingham, K.C., 1981. Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. *Journal of Biological Chemistry*, 256(23), pp.12108–12117.

Blank, W.J., He, Z.A. & Picci, M., 2002. Catalysis of the epoxy-carboxyl reaction. *Journal of Coatings Technology*, 74(926), pp.33–41.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1–2), pp.248–254.

Caballero Valdés, E., Wilson Soto, L. & Aroca Arcaya, G., 2011. Influence of the pH of glutaraldehyde and the use of dextran aldehyde on the preparation of cross-linked enzyme aggregates (CLEAs) of lipase from *Burkholderia cepacia*. *Electronic Journal of Biotechnology*, 14(3), p.10.

Devi, B.L.A.P. et al., 2009. Characterization of cross-linked lipase aggregates. *Journal of the American Oil Chemists' Society*, 86(7), pp.637–642.

Fundueanu, G. et al., 1999. Physico-chemical characterization of Ca-alginate microparticles produced with different methods. *Biomaterials*, 20(15), pp.1427–1435.

García-García, M.I. et al., 2014. First Functional and Mutational Analysis of Group 3 N-Acetylneuraminase Lyases from *Lactobacillus antri* and *Lactobacillus sakei* 23K. *PloS one*, 9(5), p.e96976.

García-García, M.I. et al., 2011. New stabilized FastPrep-CLEAs for sialic acid synthesis. *Bioresource technology*, 102(10), pp.6186–6191.

Gupta, P. et al., 2009. Characterization of cross-linked immobilized lipase from thermophilic mould *Thermomyces lanuginosa* using glutaraldehyde. *Bioresource Technology*, 100(18), pp.4074–4076.

Jasti, L.S. et al., 2014. Protein-coated polymer as a matrix for enzyme immobilization: Immobilization of trypsin on bovine serum albumin-coated allyl glycidyl ether-ethylene glycol dimethacrylate copolymer. *Biotechnology progress*, 30(2), pp.317–323.

Kim, D.K. et al., 2001. Synthesis and characterization of surfactant-coated superparamagnetic monodispersed iron oxide nanoparticles. *Journal of Magnetism and Magnetic Materials*, 225(1), pp.30–36.

Liese, A., Seelbach, K. & Wandrey, C., 2006. *Industrial biotransformations*, John Wiley & Sons.

Mateo, C. et al., 2004. A new, mild cross-linking methodology to prepare cross-linked enzyme aggregates. *Biotechnology and Bioengineering*, 86(3), pp.273–276.

Mizubuti, I.Y. et al., 2000. Response surface methodology for extraction optimization of pigeon pea protein. *Food Chemistry*, 70(2), pp.259–265.

Rodrigues, R.C., Berenguer-Murcia, Á. & Fernandez-Lafuente, R., 2011. Coupling chemical modification and immobilization to improve the catalytic performance of enzymes. *Advanced Synthesis & Catalysis*, 353(13), pp.2216–2238.

Roy, J.J. & Abraham, T.E., 2006. Preparation and characterization of cross-linked enzyme crystals of laccase. *Journal of Molecular Catalysis B: Enzymatic*, 38(1), pp.31–36.

Schoevaart, R. et al., 2004. Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs). *Biotechnology and Bioengineering*, 87(6), pp.754–762.

Shechter, L. & Wynstra, J., 1956. Glycidyl ether reactions with alcohols, phenols, carboxylic acids, and acid anhydrides. *Industrial & Engineering Chemistry*, 48(1), pp.86–93.

Sheldon, R.A., 2011. Characteristic features and biotechnological applications of cross-linked enzyme aggregates (CLEAs). *Applied microbiology and biotechnology*, 92(3), pp.467–477.

Sheldon, R.A., 2007. Cross-linked enzyme aggregates (CLEA® s): stable and recyclable biocatalysts. *Biochemical Society Transactions*, 35(6), pp.1583–1587.

Sheldon, R.A., Schoevaart, R. & van Langen, L.M., 2003. CLEAs: An effective technique for enzyme immobilisation. *Speciality chemicals*, 23(6), pp.40–42.

Sheldon, R.A., Sorgedraeger, M.J. & Kondor, B., 2012. Non-leachable magnetic cross-linked enzyme aggregate. US Patent 9,353,367

Available at: <https://www.google.es/patents/WO2012023847A2?cl=en>.

Šulek, F. et al., 2011. Immobilization of *horseradish* peroxidase as crosslinked enzyme aggregates (CLEAs). *Process Biochemistry*, 46(3), pp.765–769.

Talekar, S. et al., 2012. Bioresource Technology Novel magnetic cross-linked enzyme aggregates (magnetic CLEAs) of alpha amylase. *Bioresource technology*, 123, pp.542–547. Available at: <http://dx.doi.org/10.1016/j.biortech.2012.07.044>.

Talekar, S. et al., 2013. Carrier free co-immobilization of alpha amylase, glucoamylase and pullulanase as combined cross-linked enzyme aggregates (combi-CLEAs): a tri-enzyme biocatalyst with one pot starch hydrolytic activity. *Bioresource technology*, 147, pp.269–275.

Talekar, S. et al., 2010. Preparation and characterization of cross-linked enzyme aggregates of *Saccharomyces cerevisiae* invertase. *Aust J Basic Appl Sci*, 4(10), pp.4760–4765.

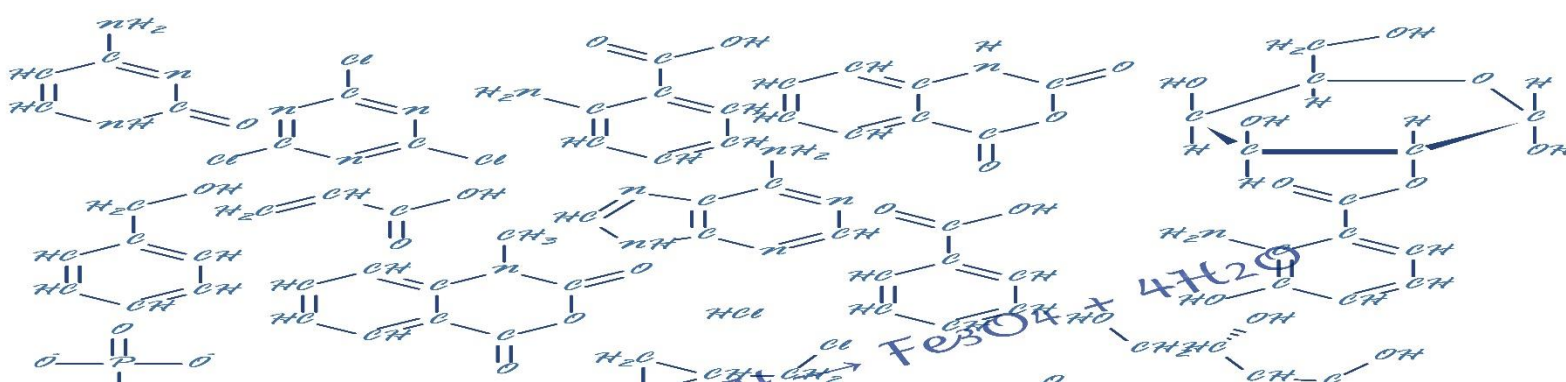
Chapter 4

Walt, D.R. & Agayn, V.I., 1994. The chemistry of enzyme and protein immobilization with glutaraldehyde. *TrAC Trends in Analytical Chemistry*, 13(10), pp.425–430.

Wilson, L. et al., 2009. Effect of the degree of cross-linking on the properties of different CLEAs of penicillin acylase. *Process Biochemistry*, 44(3), pp.322–326.

Zeeman, R. et al., 1998. Cross-linking and modification of dermal sheep collagen using 1, 4-butanediol diglycidyl ether. *J. Biomed. Mat. Res.*

Chapter 5



Epoxide-based immobilization of
Laccase inside mesocellular foam as
green catalyst for Phenoxazinone
synthesis.

Abstract

Trametes versicolor laccase was immobilized inside the pores of a mesocellular silica support, where it bound to the amino functionalized support pores by means of a triepoxide compound. The epoxide reacts with the amine groups of both the enzyme and the support and anchors the laccase to the support pores. This is the first time that glutaraldehyde (the most used cross-linked reagent in immobilization) has been replaced by a polyepoxide. Using a Box-Behnken experimental design, the immobilization variables were optimized, reaching a 90 % activity yield compared with the free enzyme. The optimized biocatalyst was tested in the oxidation-coupling formation of a phenoxazinone dye. Its properties, included temperature and pH resistance, strong affinity for the substrate 3-amino-4-hydroxybenzene sulfonate (K_M 72 μM) and good reusability (ten cycles can be performed with the minimal loss of performance) when the process was scaled up. In resume, this biocatalyst can be considered economically viable for oxidation reactions, still carried out with heavy metal.

Keywords: green synthesis; mesocellular silica support; laccase; oxidation reactions; phenoxazinones.

1 Introduction

Green catalytic alternatives are needed for oxidation processes, which tend to use inorganics compounds, such as chromium (IV), manganese dioxide, permanganate or periodate. Biocatalysts represent a promising tool for replacing chemical oxidation processes; for example, the use of enzymes avoids protection-deprotection steps, improves selectivity of the reactions and permits mild work conditions (Sheldon 2007).

Laccases are oxidoreductases present in nature in plants, bacteria, insects and fungi. They have a wide variety of substrates which they are capable of oxidizing, including phenols and their derivatives. Upon laccase-mediated oxidation, *o*-aminophenols are oxidized to the *o*-quinone-imine, a highly reactive species that can react with the reduced form to produce a phenoxazine compound. They are strong colored compounds, useful in textile dyes (Bruyneel et al. 2009), and recently tested as fluorescent dyes in live-cell imaging (Bruyneel et al. 2010). Phenoxazinones are non-toxic compounds, some of which have antibiotic and antitumor properties (Weissbach & Katz 1961) e.g. cinnabarin, actinomycin or grixazone (Fig 1).

Given these properties it would be interesting to make a robust laccase biocatalyst, destined for the industrial synthesis of phenoxazine compounds for use as pharmaceuticals, textile dyes, live-cell imaging or fluorescent probes (Newton & Milligan 2006). With this in mind, the *Trametes versicolor* laccase was immobilized in to a handmade mesoporous silica foam support, where it was anchored to the support by means of a triepoxide compound, trimethylolpropane triglycidyl ether. This represents the first report of an enzyme bind with a support by means of a polyepoxide molecule.

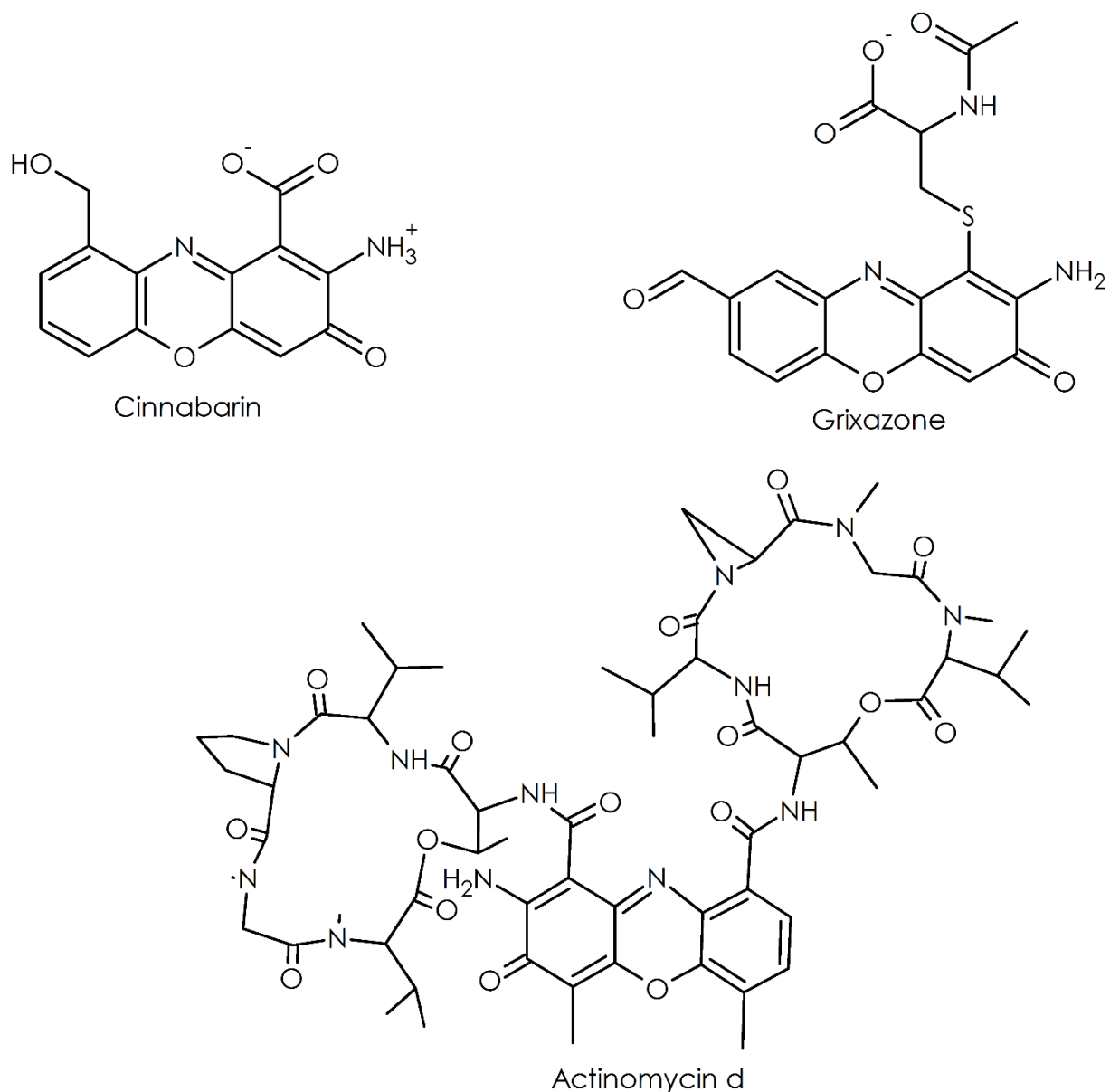


Fig 1: Phenoxiazinones structures; cinnabarin, actinomycin and grixazone

Various compounds containing epoxide groups, with different properties such as long aliphatic chains, more than two epoxide groups per molecule or different degrees of solubility in water, (features that may improve enzyme immobilization) can be used as anchor compound. Epoxides are widely used in industrial products since they are inexpensive, easy to store, and are reactive with many different functional groups such as amino, carboxyl, aldehyde, hydroxyl or thiol groups (Fraenkel-Conrat 1944), depending on the reaction conditions, however in the working conditions, the

Chapter 5

most probably reaction is with the lysine lateral amino or with a terminal amino. Such properties make them particularly suitable for fixing enzymes on supports, and for preparing biocatalysts with desirable properties (strength, hardness, stability, etc.) for use in industrial applications. Of particular note is that the bond formed between the epoxy and any group that it reacts with, is a covalent bond, which is harder than an ionic bond, or Schiff base linkage, such as that produced in the crosslinking with glutaraldehyde, the most commonly used reagent in enzyme immobilization.

2 Materials and methods

2.1 Materials

Laccase from *Trametes versicolor* (13.6 U/ mg) and trimethylolpropane triglycidyl ether were purchased from Sigma Aldrich (Sigma-Aldrich Quimica SL Madrid, Spain). AHBS (3-amino-4-hydroxybenzene sulfonate) was bought from TCI (TCI EUROPE N.V. Zwijndrecht Belgium). The rest of the chemicals used to construct the siliceous mesocellular support were also from Sigma Aldrich.

2.2 Preliminary assays

Before the laccase immobilization to the SMC foam, several preliminary assays were tested to establish the best support for the enzyme. The results were resumed and gathered in the Table 1. The supports were either prepared in our laboratory following the procedures that appear in the references of the Table 1 or purchased in Sigma Aldrich. The enzyme (1 mg/ g support) is attached to the tested supports in 10 mL acetate buffer 50 mM pH 4.5 with 5 μ L of trimethylolpropane triglycidyl ether, final concentration 1.9 mM, at room temperature with mild stirring for 12 hours. Then the biocatalysts were centrifuged (4500 \times g; 10 min; 4 $^{\circ}$ C) and washed with acetate buffer until the supernatants were clear of laccase. The supports were stocked in acetate buffer pH 4.5 at 4 $^{\circ}$ C for further analyses. The activity yield for these optimization experiments was measured using ABTS as laccase substrate.

2.3 Siliceous support preparation

Siliceous mesocellular foam (SMC Foam) was synthesized as described in the literature (Han et al. 2006). Four grams of Pluronic 123 $\text{\textcircled{C}}$ were dissolved in 75 mL 0.016 M HCl, then 3.4 mL of 1, 3, 5 trimethylbenzene was added, and the solution rapidly stirred while heating to 40 $^{\circ}$ C. After two hours, 9.2 mL of TEOS (tetraethyl orthosilicate) was added and the mixture was further heated for more than 20 hours without stirring. To the solution was added 46 mg of NH_4F in 5 mL of water and the mixture was transferred to an autoclave (100 $^{\circ}$ C) for 24 hours. The material was

filtered, washed with ethanol and dried at 60 °C in an oven. The particles were then calcinated at 550 °C in a muffle oven for 8 hours.

The resulting properties of the mesoporous siliceous support as Han et al. (2006) reported were 26.7 nm pore size, 14.5 nm window size, 535 m²/g surface area and 2.16 cm³/g pore volume.

SCM foam was functionalized by adding 500 µL of APTES (3-aminopropyl triethoxysilane) to 100 mg SMC foam, suspended in 20 mL of 70 mM phosphate buffer pH 7.0. The solution was incubated for at least 12 hours at room temperature. Then, the particles were centrifuged (4500 × g; 10 min; 4 °C) and washed with ethanol and water until no APTES was present in the supernatant (Cabana et al. 2009).

2.4 Experimental design and statistical analyses

Response surface methodology (RSM) is a statistical approach for empirical modeling which evaluates the effect of every process parameter on the corresponding response. A three level three factor Box-Behnken design was followed to optimize laccase immobilization in mesoporous silica foam and to maximize the activity yield for ABTS substrate. The chosen variables for optimization were pH in a range 3.0-6.0, laccase load (0.01-0.1 mg) and triepoxy volume (2-20 µL being the final concentration of epoxy 0.76 to 7.6 mM), the weight of SMC foam was maintained constant (100 mg) in all the assays. The experimental design and the statistical analysis were performed by STATGRAPHICS Centurion XV software version 15.2.06.

2.5 Laccase immobilization procedures

The immobilization assays were made following the above described statistical design which is showed in Table 2 and summarized the conditions of pH, enzyme load and epoxide volume used for every immobilization experiment. In general, 100 mg of siliceous material was weighed, dissolved in 50 mM acetate buffer of the required pH for the experiment (3.0-6.0) and sonicated in a water bath sonicator (Branson Ultrasonics Danbury, USA) for 30 minutes before immobilization. Then the quantity of laccase requested in the experiment was weighted and dissolved in 1 mL of the same

acetate buffer, was added and the suspension was mild stirred for two hours at room temperature. Trimethylolpropane triglycidyl ether (TMPTGE) (2-20 μL) was added and the mixture was left for 12 hours in the same conditions. The materials were centrifuged and washed with acetate buffer until the supernatants were clear of laccase. The supports were stocked in acetate buffer pH 4.5 at 4 $^{\circ}\text{C}$ for further analyses. The activity yield for these optimization experiments was measured using ABTS as laccase substrate.

2.6 Laccase activity test

2-2'-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) was used as substrate to determine laccase activity. Fifty microliters of ABTS (0.534 mM) prepared in acetate buffer (100 mM, pH 3.0) was added to 50 μL of laccase sample and 900 μL of the same buffer, in 1 mL spectrophotometric cuvette. The ABTS oxidation was monitored by measuring the absorbance at 420 nm (ϵ 36000 $\text{M}^{-1} \text{cm}^{-1}$).

All the spectrophotometric measurements were made in a Shimadzu spectrophotometer UV 2401 PC (Shimadzu Corporation Japan) equipped with a temperature controller Shimadzu TCC controller 24017.

2.7 Laccase mediated phenoxazine synthesis

Trametes versicolor laccase oxidizes 3-amino-4-hydroxybenzene sulfonate (AHBS) into the phenoxazinone yellow dye, CURIE_22, a compound with an extinction coefficient (ϵ) of 8600 $\text{M}^{-1} \text{cm}^{-1}$ (Forte et al. 2010) at 430 nm. The oxidation was monitored by recording UV/visible spectra at fixed intervals of 60 seconds. The reaction medium contained acetate buffer (50 mM, pH 4.5), 50 μg of *Trametes versicolor* laccase and 200 μM of AHBS (10 % ethanol).

2.8 Free and immobilized laccase kinetic studies for AHBS substrate

For pH and temperature optimization, 2 mM of stock AHBS solution was prepared in 10 % ethanol, the final concentration of AHBS in the cuvette being 100 μ M. The formation of a yellow compound was monitored spectrophotometrically at 430 nm. The optimum pH was measured in 50 mM acetate buffer in the range between 3.0 and 6.0 or 50 mM phosphate buffer (6.5-7.0), and the optimum temperature between 20 and 60 $^{\circ}$ C. For the kinetic assays, AHBS solutions of 0.02 to 40 mM were prepared in the same conditions as above. All the assays were performed at constant optimum temperature and pH.

The same study was made for the immobilized laccase, although in this case the biocatalyst was prepared in the optimum conditions obtained with the statistical design. The procedures were same as those used with the free laccase.

The results were adjusted to a Michaelis-Menten first order kinetic equation, while the kinetic parameters (K_M and V_{MAX}) were calculated with SigmaPlot software version 11.0.

2.9 Biocatalyst temperature stability and reusability

The temperature stability of the biocatalyst and the free laccase was measured, by incubating them at 50 $^{\circ}$ C, in acetate buffer (50 mM, pH 4.5), withdrawing aliquots at fixed intervals and measuring the activity for the phenoxazinone formation.

The process was scaled up to assess biocatalyst performance. For this 1 g of the SMC foam-laccase was prepared in the optimum conditions. The batch reaction (100 mL) was prepared in distilled water, adding the substrate to the reaction medium to reach a final concentration of 5 mM. The reaction was performed in a Metrohm 727 ti Stand device (Metrohm AG Switzerland) equipped with a water bath to maintain the temperature at 30 $^{\circ}$ C. The pH was adjusted to pH 4.5 with NaOH with a Metrohm 718 STAT titrino (Metrohm AG Switzerland). The oxygen necessary for the reaction was

Chapter 5

provided by using a high mechanical stirring rate. The biocatalyst was centrifuged and the product separated after every batch. Ten batches were performed.

3 Results and Discussion

3.1 Preliminary assays

In total five different siliceous supports were tested, for immobilization purposes. As Table 1 showed the supports with the best enzyme load are the SMC foam and the fumed silica particles prepared following the procedure of Zimmerman et al (2011). These supports have also the best activity yield, being better the fumed silica particles (92.25 %) than the SCM foam (79.59 %). However upon 7 months of storage the fumed silica particles lost more than 90 % of its activity, while the SMC foam retain almost the 90 % of it. For the high enzyme load, the good activity yield and the good storage stability the chosen support for the immobilization optimization was the handmade SMC foam.

Table 1: Siliceous support election: Preliminaries assays for Laccase covalent immobilization with TMPTGE.

Material	Size	Porous	Ref ⁶	Amine modification	Enzyme load %	A. Yield % (Fresh) ⁴	A. Yield% (Over 7 months) ⁵
Fumed silica nanoparticles	7 nm	No	(Zimmermann et al. 2011)	Yes ²	88.53	92.25	8.3
Spherical nanoparticles	1 μm	No	(Stöber et al. 1968)	Yes ²	56.87	72.29	30.91
SMC foam	5 μm	27 nm	(Han et al. 2006)	Yes ²	93.68	79.59	70.27
Silica gel¹	75-200 μm	11 nm	-	Yes ²	69.58	78.29	20.99
Silica gel¹	75-200 μm	11 nm	-	No	3.78	80.35	0.0
Silica gel functionalized¹	63 μm	No	-	Yes ³	40.36	49.64	11.73

1 Purchased from Sigma Aldrich

2 Following the method of Cabana et al. (2009)

3 Already functionalized

4 Calculated with the enzyme load

5 7 month of storage at 4 °C in acetate buffer 50 mM pH 4.5

6 Support synthesis

3.2 Optimization of Laccase immobilization

The laccase was immobilized in to amino modified siliceous mesocellular foam using a triepoxide compound as cross-linking agent, in a process optimized using Response Surface Methodology (RSM). A Box-Behnken design for three variables (pH, enzyme concentration and tri-epoxide volume) was performed using a fixed weight of SMC foam support (100 mg). Table 2 shows the data of the Response Surface Design used for optimization and the results obtained.

Table 2: Box Behnken experimental design, for the immobilization procedures, the activity Yield both experimental and predicted for the model is also show

RUN	Variables						Activity Yield ⁷	
	Coded			uncoded			Obtained	Predicted ⁸⁷
	A	B	C	pH	[Enzyme] mg/ml	[TMPTGE] μ l		
1	0	0	0	4.50	0.055	11	68.97	68.35
2	0	1	1	4.50	0.100	20	29.09	25.20
3	0	0	0	4.50	0.055	11	65.28	68.35
4	-1	-1	0	3.00	0.010	11	46.00	46.20
5	1	0	-1	6.00	0.055	2	64.23	63.85
6	0	-1	1	4.50	0.010	20	41.92	41.35
7	0	0	0	4.50	0.055	11	70.81	68.35
8	0	1	-1	4.50	0.100	2	28.51	29.08
9	-1	0	1	3.00	0.055	20	64.23	64.60
10	-1	1	0	3.00	0.100	11	27.87	31.38
11	-1	0	-1	3.00	0.055	2	91.87	87.79
12	1	1	0	6.00	0.100	11	22.95	22.75
13	0	-1	-1	4.50	0.010	2	44.00	47.89
14	1	0	1	6.00	0.055	20	72.52	76.60
15	1	-1	0	6.00	0.010	11	46.40	42.89

⁷ Measured with ABTS

⁸ Using the model described in Eq.1

The results were fitted to a second order polynomial equation (Eq. 1) to explain the relationship between the immobilization method variables and the performance of the biocatalyst. The statistical analysis data (ANOVA) for the model are presented in Table 3.

$$\text{Activity Yield} = 68.35 - 2.98 \cdot \text{pH} - 8.74 \cdot [\text{Enzyme}] - 2.61 \cdot [\text{TMPTGE}] + 2.39 \cdot \text{pH}^2 - 1.33 \cdot \text{pH} \cdot [\text{Enzyme}] + 8.98 \cdot \text{pH} \cdot [\text{TMPTGE}] - 34.94 \cdot [\text{Enzyme}]^2 + 0.67 \cdot [\text{Enzyme}] \cdot [\text{TMPTGE}] + 2.47 \cdot [\text{TMPTGE}]^2 \quad (\text{Eq.1})$$

The Regression analysis of the model showed a coefficient of determination (R^2) of 0.982, so the model explained the 98.2 % of the variability observed in the response (Activity Yield). The adjusted R^2 value (0.95) suggested the high significance of the model, the P value also showed that the model it was highly significant, because is one hundred times less that the significance coefficient (0.1). Additionally, the P value for the lack of fit was greater than 0.1, so that the model is suitable for explaining the immobilization performance with a confidence level of 90.0 %.

Table 3: Analysis of variance (ANOVA) for the proposed mathematical model (Eq.1)

ANOVA						
Source	SS	SS%	MS	F	P	df
Regression	5757.2	98	639.68	30.42	0.000767	9
Residual	105.16	2	21.03			5
LOF Error	89.30	2 (85)	29.77	3.75	0.22	3
Pure Error	15.86	0 (15)	7.93			2
Total	5862.3	100				14
R 0.991	R ² 0.982		R ² adjusted 0.950			

Having ensured that the mathematical model was able to fit the experimental data and made accurate predictions about the immobilization results, the next step was to find the optimal values for the variables in order to maximize the Activity Yield. The values that maximized the response were a pH of 3.1, an enzyme load of 0.55 mg

laccase, and 2 μL of triepoxy for 100 mg of SMC foam support. The maximum activity yield for these values is 90 %. Experimental verification of the optimum values showed an activity yield of 91.1 %, which was very close to the predicted value. The immobilization ratio was 100 %, as no enzyme was found in the supernatant.

The Response Surfaces for the variables are represented in Fig 2 to 4. As can be seen from Fig 2, which shows the relationship between the pH and the laccase concentration, the best pH for the immobilization is below 4.5 in the acidic zone, which was in good agreement with other reports (Lorenzo et al. 2005; Roy & Abraham 2006), confirmed that laccases were more stable and had better performance at acidic pH.

In the next graph (Fig 3), the influence that the volume of triepoxy had in the enzyme concentration can be seen. The enzyme shown the greatest activity yield when the volume of epoxy was maintained at low levels, probably because at the levels used there were sufficient epoxy groups for the enzyme to anchor without leaking.

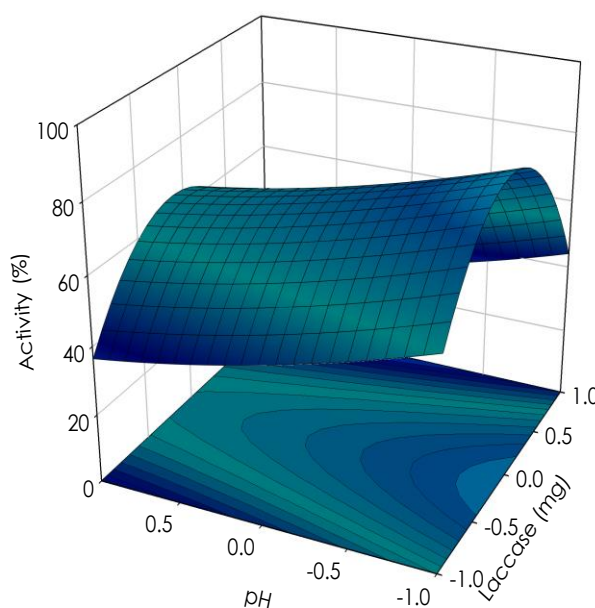


Fig 2: Response surface for the variables laccase concentration and pH, the epoxy volume is maintained in its optimum value.

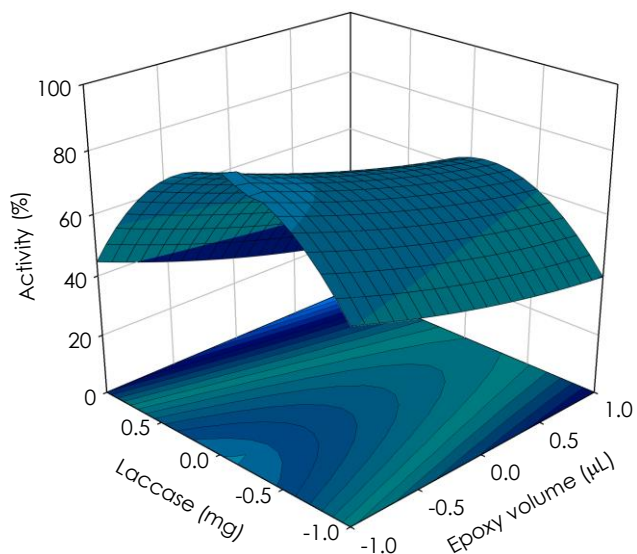


Fig 3: Response surface for the variables laccase concentration and epoxy volume, the pH is maintained in its optimum value.

Fig 4 shows the close relationship between the pH and the epoxy volume. The best performance was attained at acidic pH and low epoxy volume, nevertheless it can be seen that in mild acidic pH (5.0-6.0) the activity increased with the volume of epoxy, because the reagent was less reactive at mild pH.

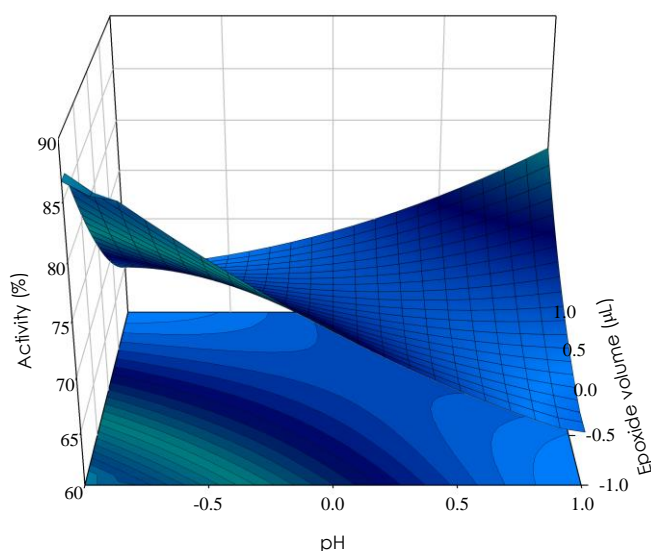


Fig 4: Response surface for the variables epoxy volume and pH, the laccase concentration is maintained in its optimum value.

3.3 Phenoxazinone compound synthesis

The compound formation is mediated with laccase and used two mol of the substrate (AHBS), for the formation of one mol of phenoxazinone (Forte et al. 2010). UV/visible spectra were recorded for the laccase mediated phenoxazinone formation as shows Fig 5. The two isosbestic points, at 280 and 300 nm, occur when two molecular entities (substrate) are converted into another (product) with a fixed stoichiometry. In these points the obtained product and the substrate have the same absorbance at a given wavelength. As long as the sum of the concentrations of the two molecular entities in the solution is held between the established stoichiometry there will be no change in absorbance at this wavelength as the ratio of the concentrations of the two entities is varied.

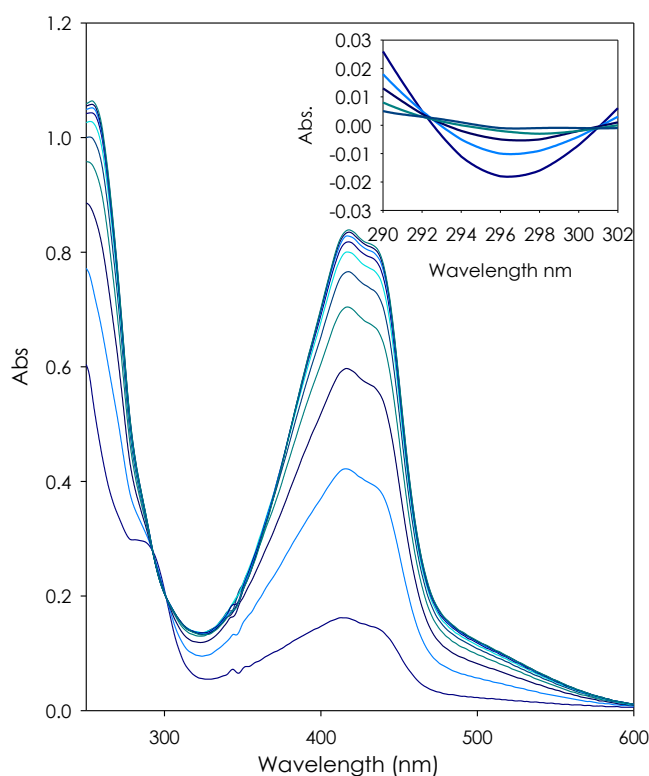
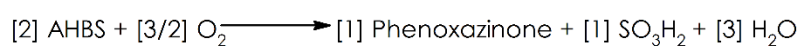
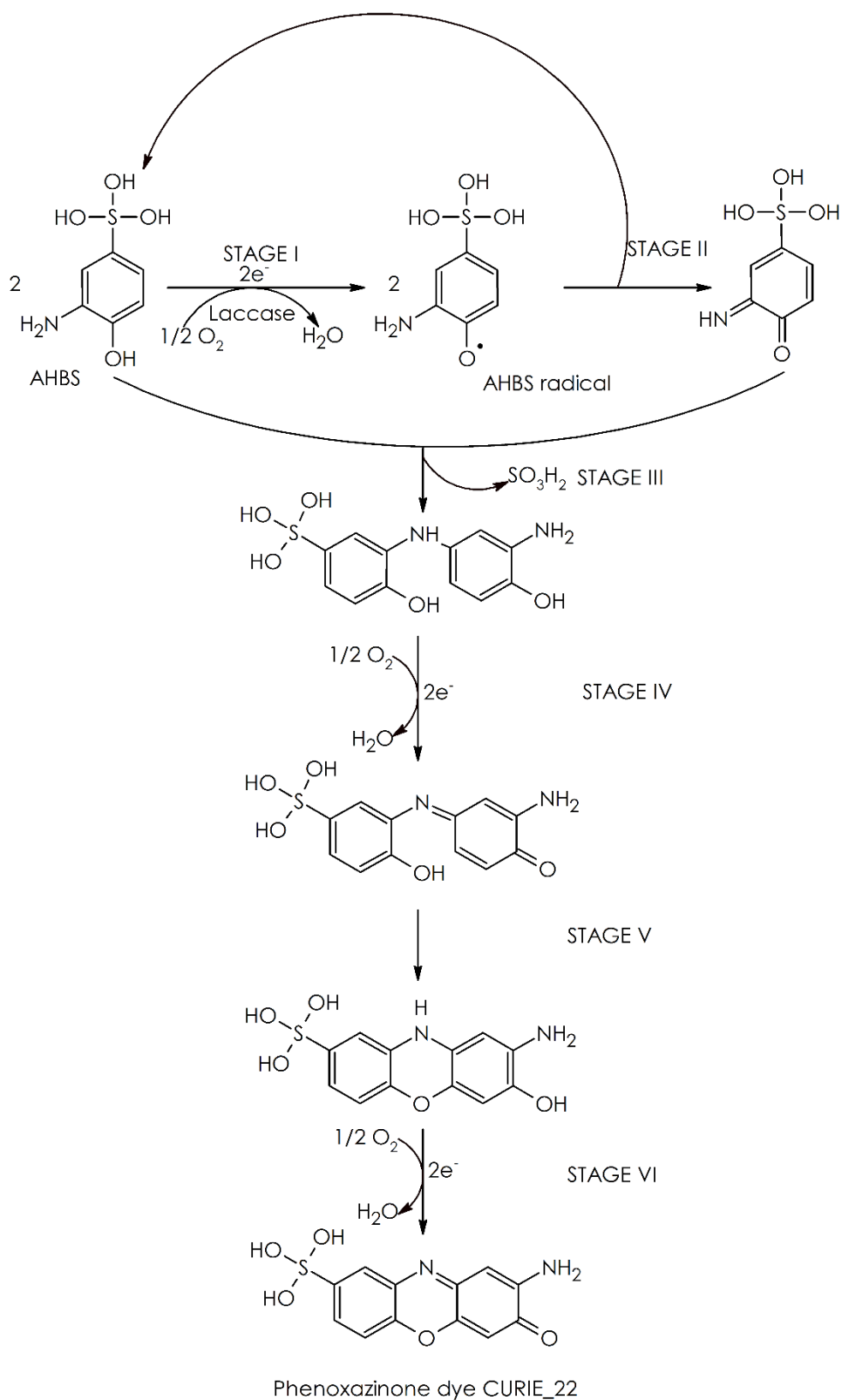


Fig 5: UV/visible spectra for laccase mediated AHBS oxidation and the phenoxazine dye formation, 200 μM AHBS and 50 μg Laccase in acetate buffer 50 mM pH 4.5. The spectra are taken at fixed intervals of 60 seconds. The **Fig 5 inset** correspond to the differential spectra of the **Fig 5**.

The observation of isosbestic points indicates that the stoichiometry of the reaction remains unchanged during the chemical reaction, and that no secondary reactions occur during the considered time range (Jiménez & García-Carmona 1999). Isosbestic points are easily detected drawing the differential spectra (Fig 5 Inset) as their absorbance is zero.

The λ_{MAX} for the phenoxazinone product is 430 nm, which was chosen for the rest of the measurements. The calculated ϵ was around $8500 \text{ M}^{-1}\text{cm}^{-1}$, which agrees with the data in the bibliography (Forte et al. 2010). The Scheme 1 shows a proposed mechanism for the coupling-oxidation reaction, being the first oxidation laccase mediated to form two molecules of AHBS radical (stage I) that dismutates in one molecule of substrate and one molecule of *o*-quinone-imine specie (II), which is coupled with one molecule of AHBS (the reduced form) to obtain a phenol-amine form (III). This coupled form will be oxidized (IV), cycled (V) and oxidized again (VI) to obtain the phenoxazinone. While the first oxidation (I) could only be enzymatic, the rest of the oxidations (IV and VI) can be enzymatic laccase mediated or chemical, mediated by the *o*-quinone-imine. By oxidizing the coupled forms the *o*-quinone-imine is reduced to substrate.



Scheme 1: Proposed scheme for the laccase mediated phenoxazinone formation

3.4 Kinetic studies

3.4.1 Optimum pH and temperature

According to Fig 6 (a), the optimum pH for both the free and the immobilized laccase was 4.5 for the AHBS substrate. The figure also shows that the support shields the enzyme against the pH; and can work in a wide pH range with low loss of activity. The optimum temperature was 30 °C (Fig 6 (b)) the same as for the biocatalyst and for the free enzyme; the biocatalyst performs better in colder temperatures whilst the free enzyme has better initial activity at higher temperatures.

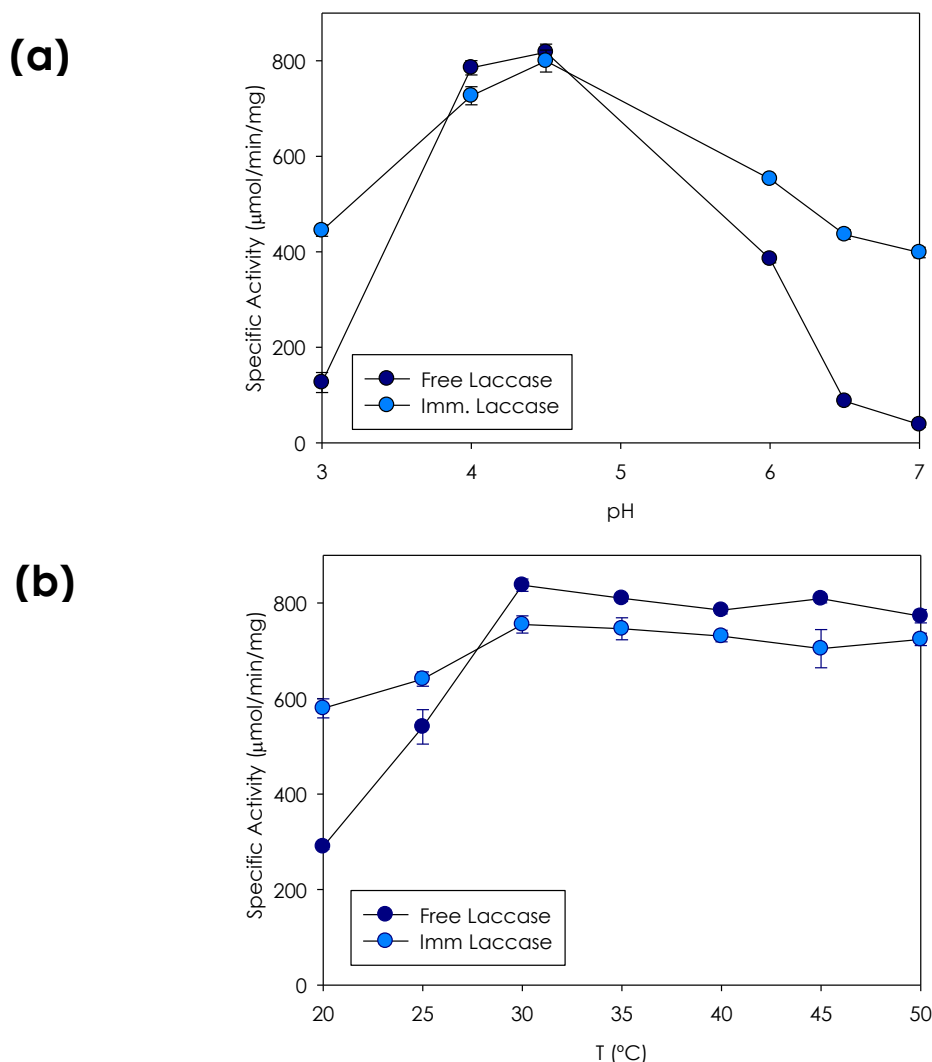


Fig 6: Comparison of: **(a)** optimum pH, the reaction medium contains 100 µL AHBS, 50 µg laccase at 25 °C for the free laccase (●) and immobilized laccase

(●) and (b) optimum temperature, the reaction medium contains 100 μL AHBS, 50 μg laccase, pH 4.5 acetate buffer 50 mM, for the free laccase (●) and immobilized laccase (●).

3.4.2 Kinetic parameters

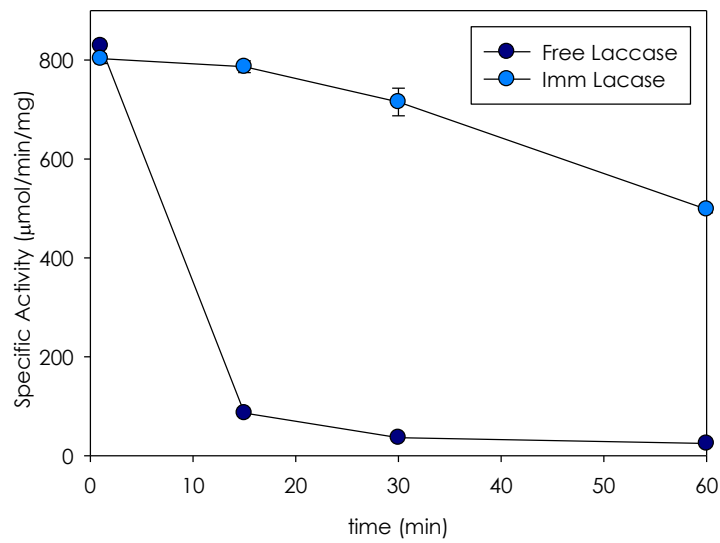
The kinetic parameters for the enzyme and the biocatalyst for AHBS substrate were calculated from Michaelis-Menten equation. The V_{MAX} for the free enzyme (14 ± 0.02 mM/min) is higher than for the immobilized enzyme (2.5 ± 0.004 mM/min), using 5 μg of free enzyme and 2 μg immobilized (that correspond to 0.5 mg of support). The K_{M} was four times lower for the immobilized enzyme (72 ± 0.6 μM) than for the free laccase (0.27 ± 0.01 mM). The epoxide groups alter the enzyme conformation in order to bind it to the support, while this bound conformation is more rigid and increases the affinity for the AHBS substrate.

3.5 Biocatalyst thermal stability and reusability

The thermal stability is show in Fig 7 (a), as can be seen the immobilization stabilized the enzyme against the temperature: whereas the free laccase lost more than the 90 % of the activity yield in the first ten minutes the immobilized enzyme only lost 2 % of the activity. This greater stabilization can be explained by the multipoint attachment that is accomplished using a triepoxide, this multipoint anchor between the epoxy, the enzyme and the amine groups preventing the enzyme denaturalization. Also the mesoporous support shields better the enzyme against the environment.

The graph drawn in Fig 7 (b) represents ten cycles of biocatalyst reuse. As can be seen, the biocatalyst had transformed more than the 95 % of the substrate into phenoxazine product in the first thirty minutes. In ten cycles of transformation, the enzyme did not lose any activity, probably due to its immobilization in the porous support. The hard bond between the epoxide groups and the enzyme permits the biocatalyst to endure tough work conditions, including high stirring speed, with no significant loss of performance.

(a)



(b)

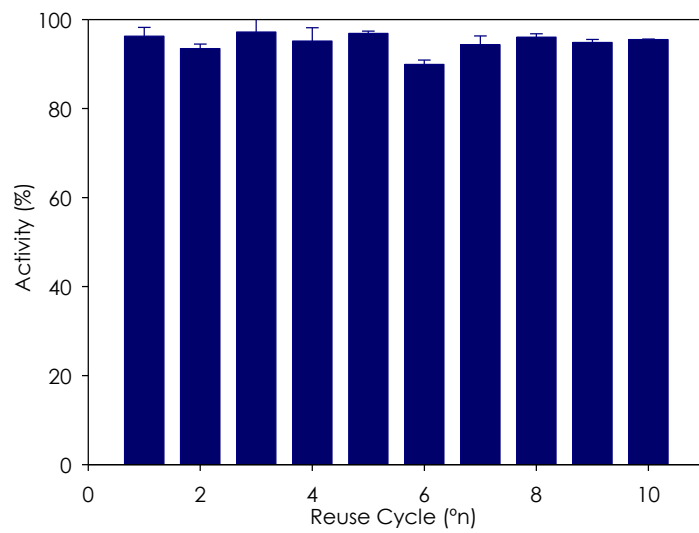


Fig 7: (a) Stability at 50 °C for the free laccase (●), and immobilized laccase (●), 50 µg laccase incubated in acetate buffer pH 4.5 50 mM and **(b)** Production cycles of AHBS coupling-oxidation in phenoxazine by the immobilized laccase (1 g), in water pH 4.5 with 5 mM of substrate.

4 Conclusions

The first step in this work was to find the best material for the immobilization, the SMC foam was chosen because it permits a high enzyme load, good activity yield (80 %), and the immobilized enzyme retains almost a 70 % of activity over 7 months of storage. Using experimental designs and Surface Response Methodology, the anchoring of *Trametes versicolor* laccase into a mesoporous silica support was optimized. The silica support was prepared and functionalized with amine groups, to which the enzyme was anchored by means of a triepoxide compound, trimethylolpropane triglycidyl ether.

The optimized biocatalyst showed an activity yield of 90 % for ABTS oxidation. The Response Surfaces study revealed that the most influential variable for this immobilization was enzyme load, which had a quadratic influence. Other investigations have also showed the importance of an optimized enzyme load (Zimmermann et al. 2011; Rekuć et al. 2010; Hommes et al. 2012). The highest activity yields usually result when the laccase load is low, because excessive laccase adsorption results in the agglomeration of enzymes inside the pores. The resulting steric constraints would subsequently restrain the dispersion and transmission of the substrate and product, which would become manifest as a reduction in activity (Wang et al. 2010).

The other two variables, pH and epoxide volume, are closely related and the epoxide group reactivity changes with changes in the pH. In mild conditions, the epoxide shows lower reactivity than in highly acidic conditions.

The immobilized laccase is a good candidate for use in green oxidation reactions, such as the production of phenoxazinone compounds, which are used in textile or pharmacological fields. Immobilization protects the enzyme against temperature denaturalization, increasing the half-life of the enzyme. Also, the support-epoxide-enzyme anchor permits several production-recovery cycles with good activity yields, with no enzyme leakage. Immobilization facilitates enzyme recovery, which is

possible after five minutes of mild centrifugation, or filtration through a glass filter to separate the biocatalyst from the reaction product. In this way, no enzyme is lost and the product is protein-free. The biocatalyst has a low K_M for the AHBS substrate, meaning that immobilization increases the affinity between the substrate and the enzyme, probably because of a conformational change in the enzyme structure caused by the epoxide and the support binding. Usually the immobilization step increases the K_M value, as reported by Roy and Abraham (2006) with the CLEC laccase or Cabana et al. (2009) with laccase immobilized onto Celite © R-633. In this case the shift in K_M value indicates a lower affinity for the substrate of the immobilized enzyme than that demonstrated by free enzyme, which might be caused by the steric hindrance, resulting in lower accessibility to the active sites of the immobilized laccase due to the covalent immobilization. The K_M for our biocatalyst was lower than that of the free laccase, since the long chains of the triepoxide and the optimized load of enzyme could reduce the steric hindrance while the bound conformation increases the affinity for the AHBS substrate. Roy and Abraham (2006) described an inactivation of *Trametes versicolor* laccase caused by high amounts of glutaraldehyde, most probably as a result of T_2 Cu depletion. This effect can be minimized by using a triepoxide for laccase immobilization, as demonstrated experiment 9 of Table 2, which involved a low pH and a high amount of triepoxide. As can be seen, the activity yield of the immobilized laccase was 64.23 %, indicating a minimized inactivation of the enzyme.

The optimized siliceous mesocellular foam-laccase biocatalyst had a 100 % immobilization rate and 90.0 % activity yield. Other attempts to immobilize *Trametes versicolor* laccase on supports such as glass beads, montmorillonite, vitroceraamics, organics gel or polyacrylamide gel, provided, in most cases, a lower immobilization rate and, frequently, a high loss of activity (Durán et al. 2002). Many of the materials used in such experiments are expensive, while porous silica is cheap, straightforward to prepare, easy to functionalize and can be stored dry for years. Also, the material is

non-toxic, has large specific surface area, presents good mechanical properties and is easy to separate from the solvent (Rekuć et al. 2010).

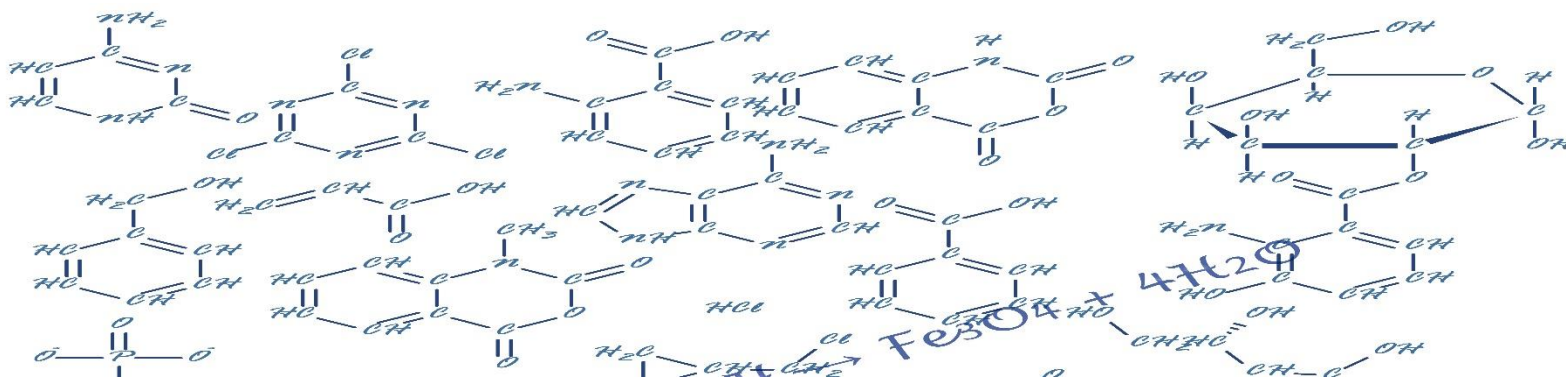
In 10 cycles of phenoxazinone production and biocatalyst recovery our immobilized laccase showed a 95 % activity yield, the activity remaining nearly unchanged for ten operating cycles. In the literature, *Panus conchatus* laccase immobilized on activated poly (vinyl alcohol) support retained 50 % of the initial activity values after 10 cycles of 2, 4, 6-trichlorophenol removal (Yinghui et al. 2002), *Trametes versicolor* laccase on mesoporous silica spheres cross-linked with glutaraldehyde retained above 70 % of activity after 10 consecutive operations (Zhu et al. 2007), immobilized *Myceliophthora thermophila* laccase on Eupergit © support retained 65 % of initial activity after 10 cycles of ABTS oxidation (Lloret et al. 2012). The only report of a laccase biocatalyst used for phenoxazinone formation was that of *Cerrena unicolor* laccase immobilized in polyacrylamide gel, when the best activity yield was 76 % in phosphate buffer (Osiadacz et al. 1999). In short, the novel method we described for binding enzymes by means of epoxides could be of great interest industrially, in the immobilization field. The laccase biocatalyst may be considered economically viable and environmentally acceptable for oxidation reactions.

5 References

- Bruyneel, F. et al., 2009. Laccase-Mediated Synthesis of Novel Substituted Phenoxazine Chromophores Featuring Tuneable Water Solubility. *Chemistry—A European Journal*, 15(33), pp.8283–8295.
- Bruyneel, F. et al., 2010. Live-Cell Imaging with Water-Soluble Aminophenoxazinone Dyes Synthesised through Laccase Biocatalysis. *ChemBioChem*, 11(10), pp.1451–1457.
- Cabana, H. et al., 2009. Immobilization of laccase from the white rot fungus *Coriolopsis polyzona* and use of the immobilized biocatalyst for the continuous elimination of endocrine disrupting chemicals. *Bioresource technology*, 100(14), pp.3447–3458.
- Durán, N. et al., 2002. Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. *Enzyme and Microbial Technology*, 31(7), pp.907–931.
- Forte, S. et al., 2010. Synthesis and structural characterization of a novel phenoxazinone dye by use of a fungal laccase. *Journal of Molecular Catalysis B: Enzymatic*, 63(3), pp.116–120.
- Fraenkel-Conrat, H., 1944. The action of 1, 2-epoxides on proteins. *Journal of biological chemistry*, 154(1), pp.227–238.
- Han, Y., Lee, S.S. & Ying, J.Y., 2006. Pressure-driven enzyme entrapment in siliceous mesocellular foam. *Chemistry of materials*, 18(3), pp.643–649.
- Hommes, G. et al., 2012. Production of a robust nanobiocatalyst for municipal wastewater treatment. *Bioresource technology*, 115, pp.8–15.
- Jiménez, M. & García-Carmona, F., 1999. Oxidation of the flavonol quercetin by polyphenol oxidase. *Journal of agricultural and food chemistry*, 47(1), pp.56–60.
- Lloret, L. et al., 2012. Immobilisation of laccase on Eupergit supports and its application for the removal of endocrine disrupting chemicals in a packed-bed reactor. *Biodegradation*, 23(3), pp.373–386.
- Lorenzo, M. et al., 2005. Inhibition of laccase activity from *Trametes versicolor* by heavy metals and organic compounds. *Chemosphere*, 60(8), pp.1124–1128.

- Newton, G.L. & Milligan, J.R., 2006. Fluorescence detection of hydroxyl radicals. *Radiation Physics and Chemistry*, 75(4), pp.473–478.
- Osiadacz, J. et al., 1999. On the use of *Trametes versicolor* laccase for the conversion of 4-methyl-3-hydroxyanthranilic acid to actinocin chromophore. *Journal of biotechnology*, 72(1), pp.141–149.
- Rekuć, A. et al., 2010. Very stable silica-gel-bound laccase biocatalysts for the selective oxidation in continuous systems. *Bioresource technology*, 101(7), pp.2076–2083.
- Roy, J.J. & Abraham, T.E., 2006. Preparation and characterization of cross-linked enzyme crystals of laccase. *Journal of Molecular Catalysis B: Enzymatic*, 38(1), pp.31–36.
- Sheldon, R.A., 2007. Enzyme immobilization: The quest for optimum performance. *Advanced Synthesis and Catalysis*, 349(8-9), pp.1289–1307.
- Stöber, W., Fink, A. & Bohn, E., 1968. Controlled growth of monodisperse silica spheres in the micron size range. *Journal of colloid and interface science*, 26(1), pp.62–69.
- Wang, F. et al., 2010. Magnetic mesoporous silica nanoparticles: fabrication and their laccase immobilization performance. *Bioresource Technology*, 101(23), pp.8931–8935.
- Weissbach, H. & Katz, E., 1961. Studies on the biosynthesis of actinomycin: enzymic synthesis of the phenoxazone chromophore. *Journal of Biological Chemistry*, 236(3), pp. 16–17.
- Yinghui, D., Qiuling, W. & Shiyu, F., 2002. Laccase stabilization by covalent binding immobilization on activated polyvinyl alcohol carrier. *Letters in applied microbiology*, 35(6), pp.451–456.
- Zhu, Y. et al., 2007. Immobilization of *Trametes versicolor* laccase on magnetically separable mesoporous silica spheres. *Chemistry of Materials*, 19(26), pp.6408–6413.
- Zimmermann, Y.-S. et al., 2011. Sorption-assisted surface conjugation: a way to stabilize laccase enzyme. *Applied microbiology and biotechnology*, 92(1), pp.169–178.

Chapter 6



Cinnabarinic acid an active compound: Characterization and Synthesis by immobilized *Trametes versicolor* laccase.

Abstract

The coupling oxidation of 3-hydroxyanthranilic acid to cinnabarinic acid has received much attention during the last few decades in clinical studies because the first is produced by interferon- γ -primed mononuclear phagocytes and seems to be involved in the protection of the mammalian tissues against the reactive oxygen species. Recently cinnabarinic acid was also studied as pharmaceutical compound for immune diseases, neurodegenerative and other neurological disorders, as well as with psychiatric diseases. This new found interest has promoted this work about a laccase biocatalyst for a sustainable obtaining of cinnabarinic acid. Substrate and product were characterized spectrophotometrically, and a reaction mechanism for the coupled-oxidation was proposed. The *Trametes versicolor* laccase cross-linked with epoxides inside the pores of the silica have 87 % activity yield for ABTS oxidation and 80 % activity yield for cinnabarinic acid formation. The biocatalyst shows good properties: good affinity for the substrate as it has a lower K_M (25 μM) than the free enzyme, good reusability (ten cycles of production-recovery without loss of activity), and good selectivity because no by-products were detected by HPLC analysis.

Keywords: cinnabarinic acid; immobilized laccase; cross-linked enzymes; siliceous mesocellular foam; phenoxazinones; oxidation reactions, biosynthesis

1 Introduction

Cinnabarinic acid (Fig 1) is an endogenous metabolite that appears in the kynurenine pathway (Scheme 1), the mayor metabolic route for the L-tryptophan degradation in the liver and other tissues in mammals, including the central nervous system (CNS).

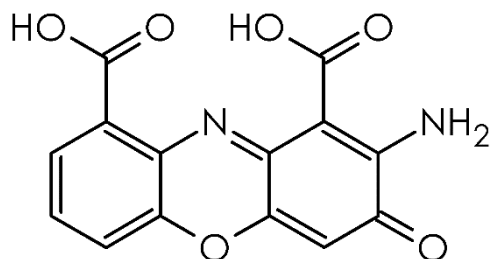


Fig 1: Cinnabarinic acid structure

Dysregulation of this pathway, results in hyper- or hypofunction of active metabolites, is associated with neurodegenerative and other neurological disorders, as well as with psychiatric diseases such as depression and schizophrenia (Schwarcz et al. 2012). Cinnabarinic acid appears as by-product of the kynurenine pathway (Scheme 1), from the condensation of two molecules of 3-hydroxyanthranilic (Fig 2) (Fazio et al. 2012).

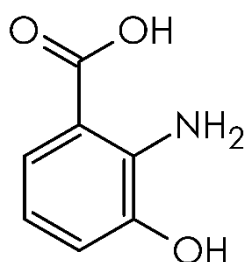
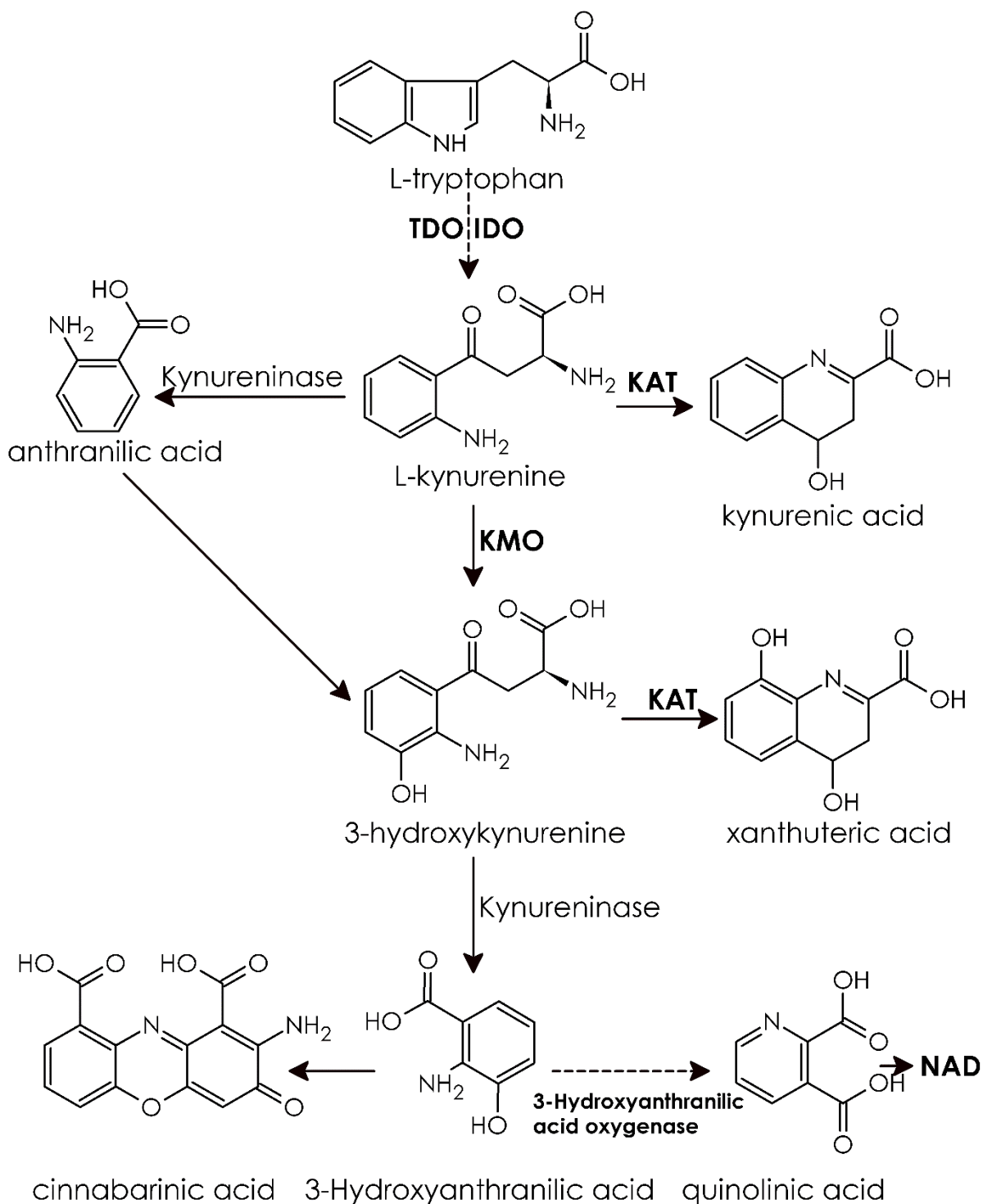


Fig 2: 3 Hydroxyanthranilic acid structure

Lately, this reagent has aroused much interest because its pharmaceutical properties. Mold (2011) has patented the use of cinnabarinic acid as modulator in autoimmune disorders, such as multiple sclerosis, systemic lupus erytromatosis,

Chapter 6

Hashimoto's disease (hypothyroiditis), Grave's disease (hyperthyroiditis), scleroderma, chronic idiopathic thrombocytopenic purpura or septic shock. (Fazio et al. 2012) tested the cinnabarinic acid as a partial agonist of mGlu4 receptors, the molecule seems to interact with the aminoacids situated in the glutamate binding pocket. They also detected a neuroprotective activity of cinnabarinic acid that was unexpected because the compound has been shown to induce apoptotic cell death in thymocytes through the generation of reactive oxygen species (ROS) and caspase activation (Hiramatsu et al. 2008). They suggested that an endogenous formation of cinnabarinic acid during neuroinflammation might protect neurons against the harmful effect of pro-inflammatory cytokines or endogenous excitotoxins. Also Stone et al. (2013) stated the relevance of these kynurenine metabolites in immune and neurological drug development and also in disorders with an inflammatory component (Darlington et al. 2010).



Scheme 1: Kynurenine pathway, from (Schwarcz et al. 2012)

The cinnabarinic acid also appears in most of the fungi as a reddish pigment with protection and antibiotic functions (Eggert 1997), is responsible of the red color of the *Pycnoporus* species, as the *Pycnoporus cinnabarius* (Fig 3) or *Pycnoporus sanguineus*. White rot fungi produce cinnabarinic acid from 3-hydroxyanthranilic acid (3-HAA) through oxidative dimerization catalyzed by laccase (EC 1.10.3.2).



Fig 3: *Pycnoporus cinnabarius* fungi

The extremely useful pharmacological properties of cinnabarinic acid have encouraged us to characterize the *Trametes versicolor* laccase mediated cinnabarinic acid formation and to prepare a robust biocatalyst for a scale up production. The support chose for the enzyme immobilization was handmade siliceous mesocellular foam derivatized with amine groups to adsorb the enzymes. Then the laccases were cross-linked with an industrial diepoxide reagent, which reacts with the amino groups of the support and with the reactive groups in the surface of the protein. The unique properties of mesoporous silica (MPS) materials were utilized to immobilize enzymes: huge surface area, modifiable surface, and restricted pore nanospaces. The enzyme confinements in the nanochannels of MPS materials generate synergistic effects that enhance enzyme stability, improve product selectivity, and facilitate separation and reuse of enzymes. Unlike sol–gel silica, mesoporous silica (MPS) materials provide tunable and uniform pore system, show biocompatibility, low cytotoxicity, large

Chapter 6

surface areas, easy functionalization, and restricted nanospaces for enzyme immobilization (Lee et al. 2009). The cross-linked with epoxides is relatively new as there are few reports of the interactions between epoxide groups with proteins (Fraenkel-Conrat 1944) and most of the reports has been towards a prepared supports functionalized with oxirane groups, such the well-known Eupergit®. Polyepoxides, as the diepoxide that was used in this work, are low cost industrial reagents with high reactivity. The reaction of the oxirane groups with the proteins can yield to a carboxylic acid esterification if they react with carboxylic acids, to a secondary amine if they react with a primary amine, to ethers with a hydroxyl or a phenolic residue, and to thio ethers with sulfhydryl groups (Fraenkel-Conrat 1944), however in the working conditions, the most probably reaction is with the lysine lateral amino or with a terminal amino. In brief, the high reactivity and the formation of covalent bonds of the epoxides and the proteins lateral chains made them perfect reagents for enzyme immobilization purposes.

2 Materials and Methods

2.1 Materials

Laccase from *Trametes versicolor* (13.6 U/ mg) and diethylene glycol diglycidyl ether were purchased from Sigma Aldrich (Sigma-Aldrich Quimica SL Madrid, Spain). 3-HAA (2-Amino-3-hydroxybenzoic acid/ 3-Hydroxyanthranilic acid) was bought from TCI (TCI EUROPE N.V. Zwijndrecht Belgium). The rest of the chemicals used to prepare the siliceous mesocellular support were also from Sigma Aldrich.

2.2 Methods

2.2.1 Siliceous support preparation

Siliceous mesocellular foam (SMC Foam) was synthesized as described in the literature (Han et al. 2006). Four grams of Pluronic 123[®] were dissolved in 75 mL 0.016 M HCl, then 3.4 mL of 1, 3, 5 trimethylbenzene was added, and the solution rapidly stirred while heating to 40 °C. After two hours, 9.2 mL of TEOS (tetraethyl orthosilicate) was added and the mixture was further heated for more than 20 hours without stirring. To the solution was added 46 mg of NH₄F in 5 mL of water and the mixture was transferred to an autoclave (100 °C) for 24 hours. The material was filtered, washed with ethanol and dried at 60 °C in an oven. The particles were then calcinated at 550 °C in a muffle oven for 8 hours.

SCM foam was functionalized by adding 500 µL of APTES (3-Aminopropyl triethoxysilane) to 100 mg SMC foam, suspended in 20 mL of 70 mM phosphate buffer pH 7.0. The solution was incubated for at least 12 hours at room temperature. Then, the particles were centrifuged (4500×g; 10 min; 4 °C) and washed with ethanol and water until no APTES was present in the supernatant (Cabana et al. 2009).

2.2.2 Laccase immobilization procedure

One hundred milligrams of material was weighed, dissolved in 10 mL acetate buffer (50 mM pH 4.5) and sonicated for 30 minutes before immobilization. Then 1 mL of enzyme (0.5 mg/ mL) also dissolved in acetate buffer, was added and the suspension was mild stirred for two hours at room temperature. Diethylene glycol diglycidyl ether (100 μ L) was added and the mixture was left for 12 hours in the same conditions. The materials were centrifuged (4500 \times g; 5 min; 4 $^{\circ}$ C) and washed with acetate buffer until the supernatants were clear of laccase. The supports were stocked in acetate buffer pH 4.5 at 4 $^{\circ}$ C for further analyses.

2.2.3 Laccase activity test

2-2'-Azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) was used as substrate to determine laccase activity. Fifty microliters of ABTS (0.534 mM) prepared in acetate buffer (100 mM, pH 4.5) was added to 50 μ L of laccase sample and 900 μ L of the same buffer, in 1 mL spectrophotometric cuvette. The ABTS oxidation was monitored by measuring the absorbance at 420 nm (ϵ 36000 M⁻¹ cm⁻¹).

All the spectrophotometric measurements were made in a Shimadzu spectrophotometer UV 2401 PC (Shimadzu Corporation Japan) equipped with a temperature controller Shimadzu TCC CONTROLLER 24017.

2.2.4 Laccase mediated cinnabarinic acid synthesis

Trametes versicolor laccase oxidizes 3-hydroxyanthranilic acid (3-HAA) into the phenoxazinone cinnabarinic acid, a compound with an extinction coefficient (ϵ) that is pH dependent and had a maximum absorption wavelength at 450 nm. The formation of cinnabarinic acid was monitored by recording UV/visible spectra at fixed intervals of 90 seconds. Assays were prepared in a 1 mL spectrophotometric cuvette. The reaction medium contained acetate buffer (50 mM, pH 5.0), different enzyme weight

0.4 μg , 2 μg , 10 μg of *Trametes versicolor* laccase and different concentrations of 3-HAA 36 μM , 120 μM , 1 mM (being the final concentration of methanol 1 %) at 25 °C without stirring. The concentration of oxygen in the solution was supposed 180 μM .

2.2.5 Cinnabarinic acid formation with periodate

The coupling oxidation reaction to obtain cinnabarinic acid was simulated with sodium periodate, a chemical oxidant. As in the reaction with laccase the reaction medium contained acetate buffer (50 mM, pH 5.0) 3-hydroxyanthranilic acid 200 μM and different molar rates of sodium periodate (10:1 and 1:1) were added to it. The oxidation was monitored by recording UV/visible spectra at fixed intervals of 90 seconds.

2.2.6 Coleman analysis

Coleman graphical analysis was made following the method that Coleman (Coleman et al. 1970) reported. For the analysis of two species with restrictions A_{ij} is the absorbance at the selected wavelength i at recording j , i' 450 nm and j' the first record, the selected wavelengths (i) are 290, 300, 315, 330, 400, 500, 550 nm. The analysis of three species with restrictions, m is 315 nm, n is 450 nm, j' the first record and the selected wavelengths (i) are 290, 300, 330, 400, 500, 550 nm.

2.2.7 3-Hydroxyanthranilic acid and Cinnabarinic acid pH dependence studies

Substrate 3-hydroxyanthranilic acid and the product cinnabarinic acid were submitted to a pH study. 2 mM substrate stock solution was prepared in 10% ethanol, and 50 μL of this solution was mixed with acetate buffer (50 mM, pH 3.0-6.0) or phosphate (50 mM, pH 6.5-7.0) in a 1 mL quartz cuvette, UV/visible spectra were recorded at different pH in the range between 3.0 and 7.0. The pK_a for the substrate was calculated with SigmaPlot software version 11.0. It was obtained by representing

the absorbance values at maximum wavelength respect to pH and adjusting the data to a sigmoidal function.

The cinnabarinic acid was obtained by laccase oxidation at different pH with different substrate amounts and an excess of enzyme to assure the depletion of the substrate. The absorbance values at 450 nm were adjusted to the Lambert-Beer equation $A = \epsilon \cdot b \cdot c$ being b 1 cm, the extinction coefficient (ϵ) was calculated for each pH. Then the calculated extinction coefficients (ϵ) were represented versus their pH and adjusted to a sigmoidal function, in order to obtain the cinnabarinic acid pKa.

2.2.8 Free and immobilized laccase kinetic studies for 3-Hydroxyanthranilic acid

For pH and temperature optimization, 2 mM of stock 3-HAA solution was prepared in 10% ethanol, the final concentration of 3-HAA in the cuvette being 100 μ M, and the formation of a redish-yellow (cinnabarinic acid) compound was monitored spectrophotometrically at 450 nm. The optimum pH was measured in acetate buffer (50 mM) in the range between 3.0 and 6.0, and in phosphate buffer (50 mM) for pH 6.5 to 7.0 at 25 °C. The optimum temperature was obtained in the interval of 20 to 70 °C using the obtained optimal pH. For the kinetic assays, 3-HAA solutions of 0.02 to 40 mM were prepared in the same conditions as above. All the assays were performed at constant temperature and pH. The same study was made for the immobilized laccase, using the same enzyme mass as in the assays with the free enzyme.

The results were adjusted to a Michaelis-Menten first order kinetic equation, while the kinetic parameters (K_M and V_{MAX}) were calculated with SigmaPlot software version 11.0.

2.2.9 Biocatalyst reusability

The process was scaled up to assess biocatalyst performance. For this, 1 g of the SMC foam-laccase was prepared in the same conditions. The batch reaction (100 mL) was prepared in distilled water, adding the substrate to the reaction medium to reach a final concentration of 2.5 mM. The reaction was performed in a Metrohm 727 ti Stand device (Metrohm AG, Switzerland) equipped with a water bath to maintain the temperature at 25 °C. The pH was adjusted to pH 3.0 with NaOH with a Metrohm 718 STAT titrino (Metrohm AG, Switzerland). The oxygen necessary for the reaction was provided by using a high mechanical stirring rate. The biocatalyst was centrifuged and the product separated after every batch. Ten cycles were performed.

2.2.10 Cinnabarinic acid HPLC analysis

Aliquots were taken of one of the production batches at fixed time intervals the samples were centrifuged at 13000 rpm for ten minutes, and then submitted to HPLC analysis, in a Shimadzu prominence modular HPLC (Shimadzu Corporation, Tokyo Japan), equipped with a CHROMPACK C18 silica column (250×4.6 mm) (Varian, Inc.). The mobile phase was linear gradient of methanol (0.086 % v/v phosphoric acid) starting in 5 % and ending in 100 % of methanol, being the flow rate 0.5 mL/min.

3 Results and Discussion

3.1 Laccase mediated cinnabarinic acid synthesis

The repetitive spectra for the coupling-oxidation for 3-HAA and the formation of cinnabarinic acid, with a high substrate concentration (1 mM), excess of substrate in relation to soluble oxygen (180 μ M) were shown in Fig 4 (a). The spectra analysis can be divided in two stages. In the first (spectra 1-10) the substrate was oxidized to imino-quinone by laccase with oxygen, and then coupled with another 3-hydroxyanthranilic molecule, that was oxidized, cycled and oxidized again to give cinnabarinic acid, no isosbestic point can be detected. The second stage (spectra 11 to 20) is marked for the apparition of an isosbestic point and an oxygen depletion, therefore the enzymatic reaction was stopped, the reaction continues only with the imino-quinone to produce cinnabarinic acid. The Fig 4 (a) inset shows the differential spectra of the isosbestic point. The isosbestic point indicated the presence of two absorbents species whom molar relation does not varies with the time. Coleman graphical analysis support the presence of two absorbent species kinetically related (Fig 1s of supplementary material), imino-quinone and final product cinnabarinic acid. The continuous formation of product in anoxia conditions suggested that the formation of the imino-quinone was oxygen dependent but the oxidation of the leuco form to orthoquinone and it posterior stabilization in paraquinone were not, and can be attained by reduction of the imino-quinone to 3-hydroxyanthranilic acid. That implies a constant stoichiometry of three imino-quinone molecules to give one cinnabarinic acid molecule.

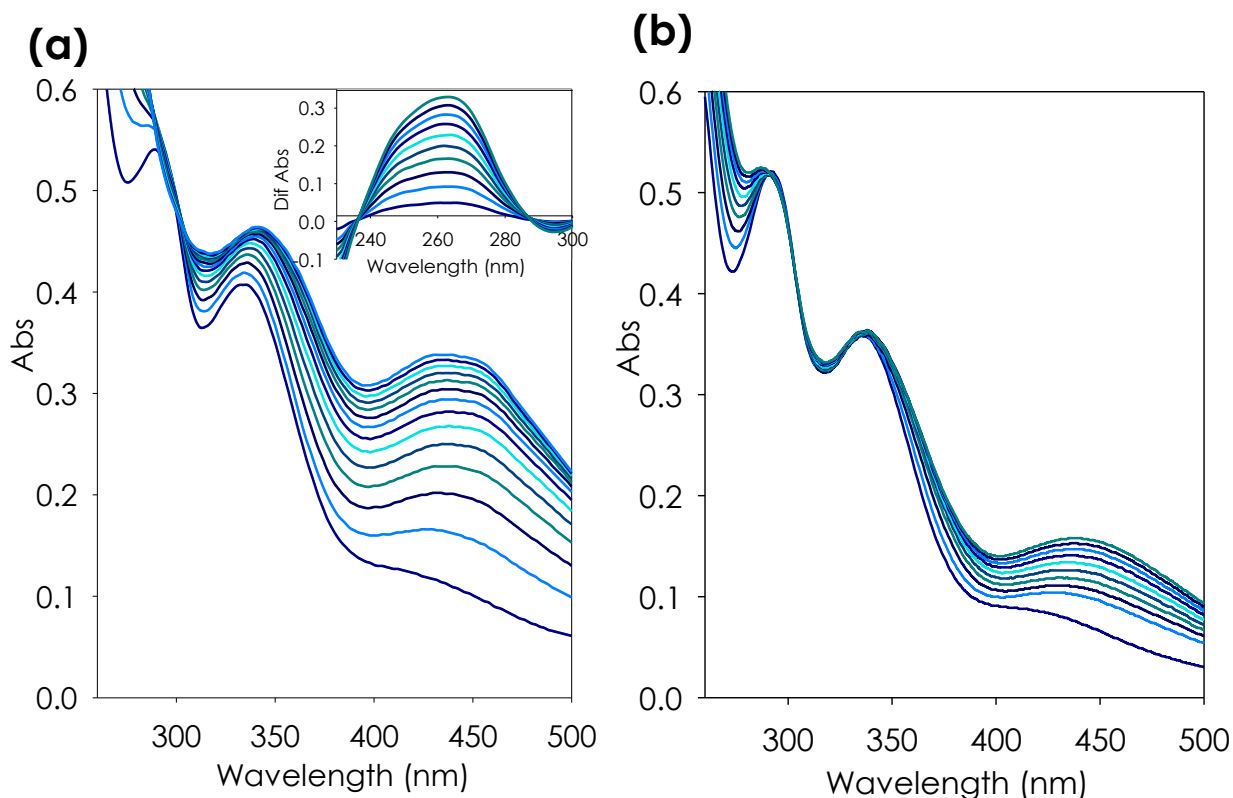


Fig 4: (a) Repetitive UV/visible spectra for the laccase mediated cinnabarinic acid production with substrate excess, 1 mM 3-HAA and 2 μg laccase in acetate buffer at pH 5.0 and 25 $^{\circ}\text{C}$. The first spectrum corresponds to the substrate (3-HAA) at pH 5.0. The differential spectra are show in the **inset**. (b) Repetitive UV/visible spectra for the sodium periodate oxidation in equimolar concentrations of reagents, 200 μM 3-HAA and 200 μM sodium periodate in acetate buffer at pH 5.0 and 25 $^{\circ}\text{C}$. The differential spectra are show in the insert.

Reaction in low enzyme and substrate concentration was illustrated in Fig 2s of the supplementary material, the Coleman analysis gives a good adjust for two species (Fig 3s), however no isosbestic point is detected in the spectral range studied. In this situation the cinnabarinic acid formation was lineal with the time but very slow, because there was few imino-quinone accumulated. When increasing the laccase

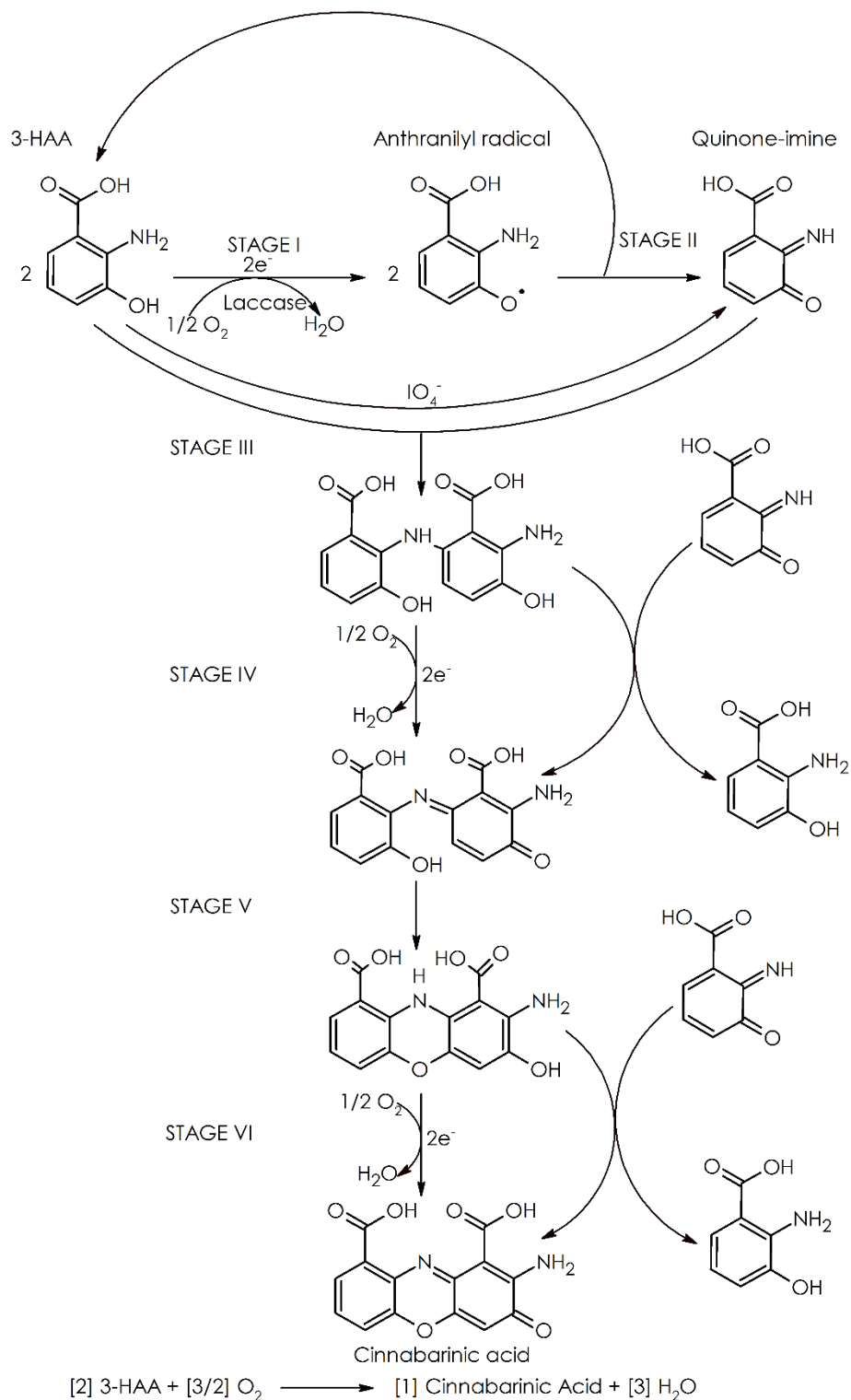
quantity (Fig 4s) a fast transformation of substrate to a colored compound (imino-quinone) was shown, this compound was not cinnabarinic acid. This intermediate was fast transformed to cinnabarinic acid, but once the accumulated imino-quinone was consumed the transformation rate of the product is slowed down almost stopped.

In stoichiometric concentrations of oxygen and substrate, the spectra do not have an isosbestic point (Fig 5s), but by Coleman analysis (Fig 6s) two kinetically related species were obtained. As can be seen, in the first stages of the reaction the formation of substrate is faster than in the next stages, most probably produced by the excess of oxygen, when the oxygen concentration decreases the reaction rate slows.

The oxidation of 3-HAA to give cinnabarinic was simulated with sodium periodate. When substrate- periodate were in equimolar quantities as illustrated in Fig 4 (b), a large extent of substrate was transformed into imino-quinone. Then the imino-quinone was coupled with substrate to form cinnabarinic acid in a fast reaction as can be seen in the second spectrum of Fig 4 (b). The reaction was then slowed down since there is less oxidant and less substrate to be oxidized and coupled, and the reaction becomes linear with time. This marks the coupling of imino-quinone and substrate as the slow reaction since it is dependent of the availability of substrate and the formation of imino-quinone. The visualization of isosbestic point and the Coleman analysis (Fig 7s) shows a two species kinetically related with a constant molar relationship.

In excess of periodate (Fig 8s), there was a fast formation of a colored compound, imino-quinone, which evolves slowly with time. The substrate is completely oxidized to imino-quinone and the reaction was stopped as there was no substrate to be coupled with it. The Coleman analysis does not adjust to two species but a three species (Fig 9s) indicative of imino-quinone evolution to other species (e.g. superoxide or dimeric forms) in excess of oxidant as reported before (Iwahashi, 1999).

The proposed scheme for cinnabarinic acid production was depicted in Scheme 2, the scheme showed that with laccase the anthranilyl radical can be formed (STAGE I), since laccases can remove one electron at time and generate free radicals (Torres-Pacheco et al. 2006). This unstable radical dismutates in one molecule of substrate and one molecule of *o*-imino-quinone (STAGE II). Using periodate as oxidant this is not possible, as periodate will oxidized the molecule until the *o*-imino-quinone (STAGE II). The coupling of the imino-quinone and substrate (STAGE III) was supposed to be the rate determining step, as depended of the concentration of substrate and the formation of imino-quinone. If there was excess of oxidant the majority of substrate will be oxidized to imino-quinone, few substrate will be left to the coupling reaction, and the formation of cinnabarinic acid will be slow. The same occurs in defect of oxidant, little imino-quinone will be produced and the coupling will be slow. The repetitive spectra in excess (Fig 8s), and equimolarity (Fig 4 (b)) of oxidant and substrate suggested that once the leuco form was produced (STAGE IV) the formation of orthoquinone (STAGE V) and the stabilization in paraquinone (STAGE VI), can be achieved by reduction of the imino-quinone to 3-hydroxyanthranilic acid as was reported for dopaquinone (Jimenez et al. 1984; Garcia-Cánovas et al. 1982). The formation of cinnabarinic acid can be measured at 450 nm, as the spectra shows in Fig. 4 (a) and 4 (b).



Scheme 2: Coupling-oxidation proposed mechanism for cinnabaric acid enzymatic formation, a modification of the scheme proposed by Iwahashi (1999).

3.2 3-Hydroxyanthranilic and Cinnabarinic acid pH dependence studies

The pH dependence of 3-hydroxyanthranilic acid is represented in Fig 5, it shows that the spectra shape changes when changing the pH, at acid pH spectra show two maximum that bind into one when the media is neutralized. The presence of two isosbestic points at 290 and 340 nm indicates that the compound have pKa in the pH range. In the insert the maximum absorbance is represented respect pH. The representation was adjusted to a sigmoidal function and a pKa value of 5.05 was obtained with regression coefficient of 0.9979.

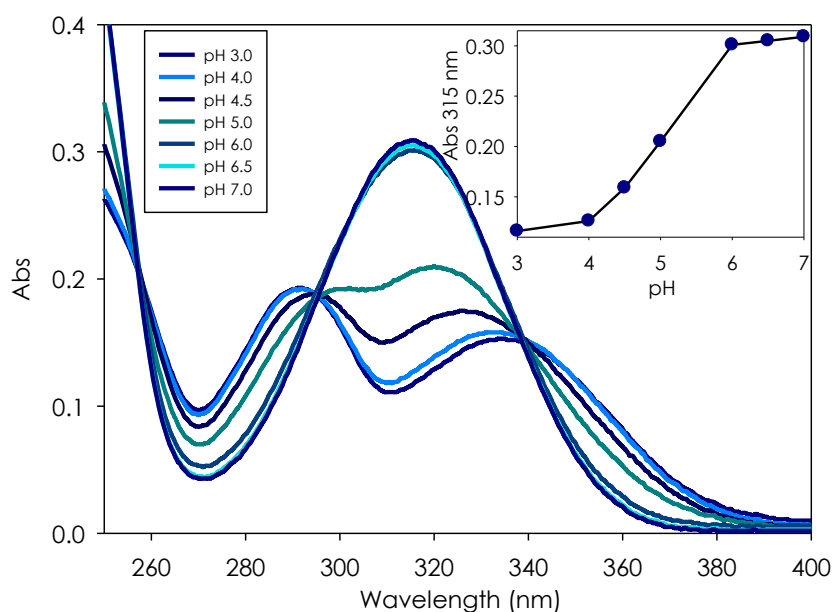


Fig 5: 3-Hydroxyanthranilic acid [100 μ M] spectra in acetate/ phosphate buffer (50 mM) at different pH between 3.0 and 7.0 and 25 °C. The insert shows the spectrum data adjusted to a sigmoidal curve to obtaining 3-Hydroxyanthranilic acid pKa.

Cinnabarinic acid has also a pKa in the pH range, the shape of the spectra does not change while changing the pH, however the extinction coefficient (ϵ) of this compound is pH dependent indicating a pKa in the pH range. In order to obtain the

extinction coefficients, kinetic assays with different substrate concentration were made for each pH, for example, in Fig 6 the laccase kinetic for cinnabarinic acid formation at pH 4.5 and 25 °C with different 3-HAA concentrations is presented, when the reaction is completed the maximum absorbance is adjusted with the Lambert-Beer law.

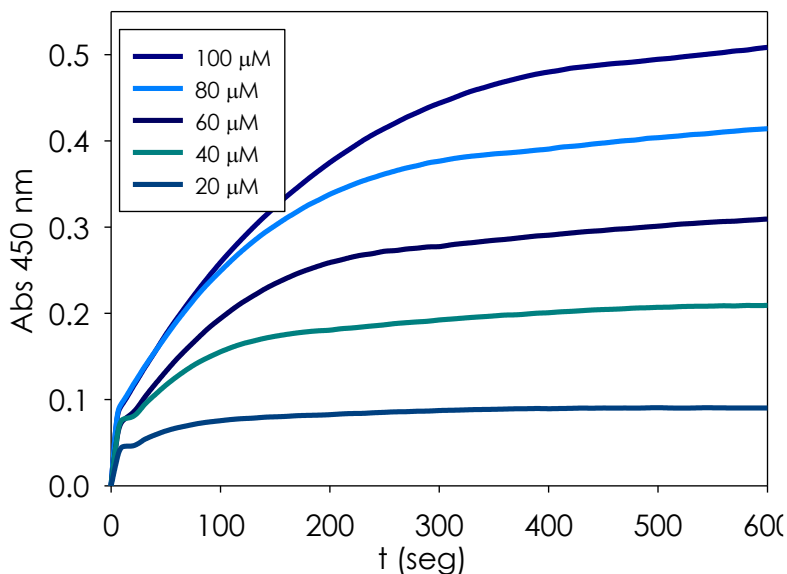


Fig 6: Laccase kinetic curves with different molar concentrations of 3-Hydroxyanthranilic acid at pH 4.5 and 25 °C, used to estimate the cinnabarinic acid molar extinction coefficient for pH 4.5.

These experiments were made for each pH and the obtained extinction coefficients (ϵ) were gathered in Table 1. The adjusted data confirms the reaction stoichiometry two moles of 3-hydroxyanthranilic acid gives one mol of cinnabarinic acid, as shows Scheme 2. As Table 1 shows the molar extinction coefficient for the product increases with increasing the pH, probably because one of the groups which are involved in the molecule color gets protonated in acidic pH provoking a diminution in the resonance (Chandrasekaran 2001), the same occurs with the substrate, as can be seen in Fig 5, the maximum absorbance increase when increasing the pH. By adjusting the pH and the extinction coefficients to a sigmoidal function, the pKa for the cinnabarinic acid is also obtained (pKa 4.33).

Table 1 Extinction Coefficient for Cinnabarinic acid at different pH, at 25 °C.

pH	ϵ ($M^{-1}cm^{-1}$)	R^2
3	3717	0.9998
4.5	10265	0.9988
5	12957	0.9992
6	17093	0.9996
7	17968	0.9980
pKa	4.33 \pm 0.079	R^2 0.9984

3.3 Laccase immobilization

The laccase immobilization in the siliceous mesocellular foam support following the procedure established in the section 2.2.4 of Materials and Methods, yields to 87.12 % activity yield for ABTS oxidation and 100 % immobilization rate. The loss of activity yield could be due diffusional limitations, which can be minimized with a high stirring speed (Klibanov 1997). The properties for this mesoporous siliceous support as (Han et al. 2006) reported are 26.7 nm pore size, 14.5 nm window size, 535 m²/g surface area and 2.16 cm³/g pore volume. Immobilization facilitates the enzyme recovery, which is possible with five minutes of mild centrifugation, or filtration through a glass filter to separate the biocatalyst from the reaction product. In this way no enzyme is lost and the product is protein free. Being laccases useful enzymes for oxidation reactions in the literature there are many examples of laccase immobilization, like (Lu et al. 2007) that immobilized laccase into alginate-chitosan microcapsules resulting in 88.12 % loading efficiency and 46.93 % activity yield, (Dai et al. 2010) encapsulated in situ *Trametes versicolor* Laccase into the poly(D,L-lactide) (PDLLA)/PEO-PPO-PEO (F108) electrospun microfibers by emulsion electrospinning, the enzyme retain 67 % activity yield, or (Lloret et al. 2012) that immobilized *Myceliophthora thermophila* laccase onto Eupergit® (a commercial epoxide modified support) reaching activity yields between 36.1 % and 88.4 %. Many of the materials

used in such experiments are expensive, while the porous silica is cheap, easy to prepare, easy to functionalize and can be stored dry for years. Also, the material is non-toxic, has large specific surface area, presents good mechanical properties and is easy to separate from the solvent (Rekuć et al. 2010).

3.4 Free and immobilized laccase kinetic studies for 3-Hydroxyanthranilic acid

The laccase activity towards the cinnabarinic acid was pH-dependent as shows Fig 7, the optimum pH for the free laccase is 4.5, value that is in agree with others laccase reports (Lorenzo et al. 2005; Roy & Abraham 2006), for the immobilized enzyme the best pH is 3.0. There is a pH shift between the native laccase and the biocatalyst. Probably caused by the amines from the APTES that are used to functionalized the support pores, the amines gets protonated and modify the pH inside the pores, the pH in the pores increases and the optimum pH (measured as external pH) for the enzyme decreases to maintain the best pH for the enzyme performance. This proton gradient inside the pores caused by the static charges on the support was also reported by others authors (Lee et al. 2009; Tischer & Wedekind 1999). The optimum temperature was maintained at 25 °C, to avoid the possible degradation of the product.

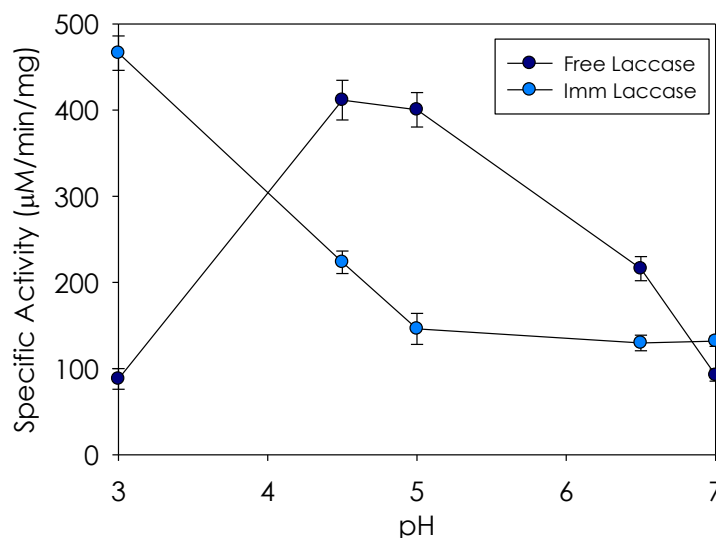


Fig 7: Free (●) and immobilized (●) laccase optimum pH for the substrate 3-Hydroxyanthranilic acid, measured in acetate buffer (50 mM pH 3.0-6.3), or phosphate buffer (50 mM pH 6.5-7.0), 100 µM of 3-HAA, 50 µg laccase.

The Michaelis-Menten kinetic parameters for the substrate 3-hydroxyanthranilic acid are gathered in Table 2. As the Table shows, the V_{MAX} is higher for the free laccase than for the immobilized. The K_M value is fourfold lower for the immobilized laccase than for the free laccase, meaning that the immobilization procedures improves the enzyme affinity for the substrate. The epoxide binds the laccase in a better structural conformation for the oxidation of 3-hydroxyanthranilic acid, also the reaction inside the siliceous pores promotes more selectivity towards this substrate. Specific activity shows that the activity yield for the immobilized enzyme is the 80 % than the free enzyme for the 3-hydroxyanthranilic substrate.

Table 2 Kinetic parameters of 3-hydroxyanthranilic acid substrate for the free and the immobilized laccase, 25 °C, pH 4.5 for free laccase and pH 3.0 for the immobilized one.

	V_{MAX} (mM/min)	K_M (mM)	Specific Activity (μ M/min/mg)
Free Laccase	0.040 \pm 0.01	0.10 \pm 0.01	810
Immobilized Laccase	0.024 \pm 0.002	0.025 \pm 0.002	640

3.5 Biocatalyst reusability

A scale up process was made to test if the biocatalyst accomplishes the industrial requirements, like good performance, stability and reusability. Fig 8 (a) shows ten cycles of cinnabarinic acid production-recovery, the production yield reach the 95 % in one hour at 25 °C, pH 3.0 without buffered medium. The biocatalyst maintain the activity in the ten cycles with no significant loss of performance, the hard bond form between the protein and the support provided by the diepoxide cross-linking avoids the enzyme leakage and the situation of the enzyme inside the pores shields it against the work conditions such as the stirring speed or the reagents used to maintain the pH (NaOH). The product obtained in one of these cycles was used for the HPLC analysis reported below. In literature, *Trametes versicolor* laccase on mesoporous silica spheres cross-linked with glutaraldehyde retained above 70 % of activity after 10 consecutive operations (Zhu et al. 2007), immobilized *Myceliophthora thermophila* laccase on Eupergit® support retained 65 % of initial activity after 10 batches for ABTS oxidation Lloret et al. (2012).

3.6 HPLC analysis

Aiming to characterize better the biocatalyst in a scale up process aliquots were withdrawn, at fixed time intervals, in one of the production cycles and were submitted to HPLC analyses, the obtained chromatograms are show in Fig 8 (b). The first peak belongs to the substrate 3-hydroxyanthranilic acid, the peak high decreases as the reaction time advances while the second peak belonging to the product cinnabarinic acid increases. When the reaction is complete (120 min) only the second peak is detected, meaning than the substrate and the reaction intermediates have been transformed to the main product. Doing the same scale up experience with the free enzyme buffered at the optimum pH (4.5) more peaks between the substrate and the product could be detected (data not show), probably belonging to a stable intermediates (i.e. a stabilized quinone) or a trimeric structure of substrate. Also the cause of by-products could be the buffered solution (Bruyneel et al. 2008) reported a high formation of by-products when they scale up the process using buffered mediums so they decided to adjust the pH by the addition of HCl/NaOH in order to decrease the by-products formation. In resume, the biocatalyst improves the selectivity of the enzyme and only releases the main product.

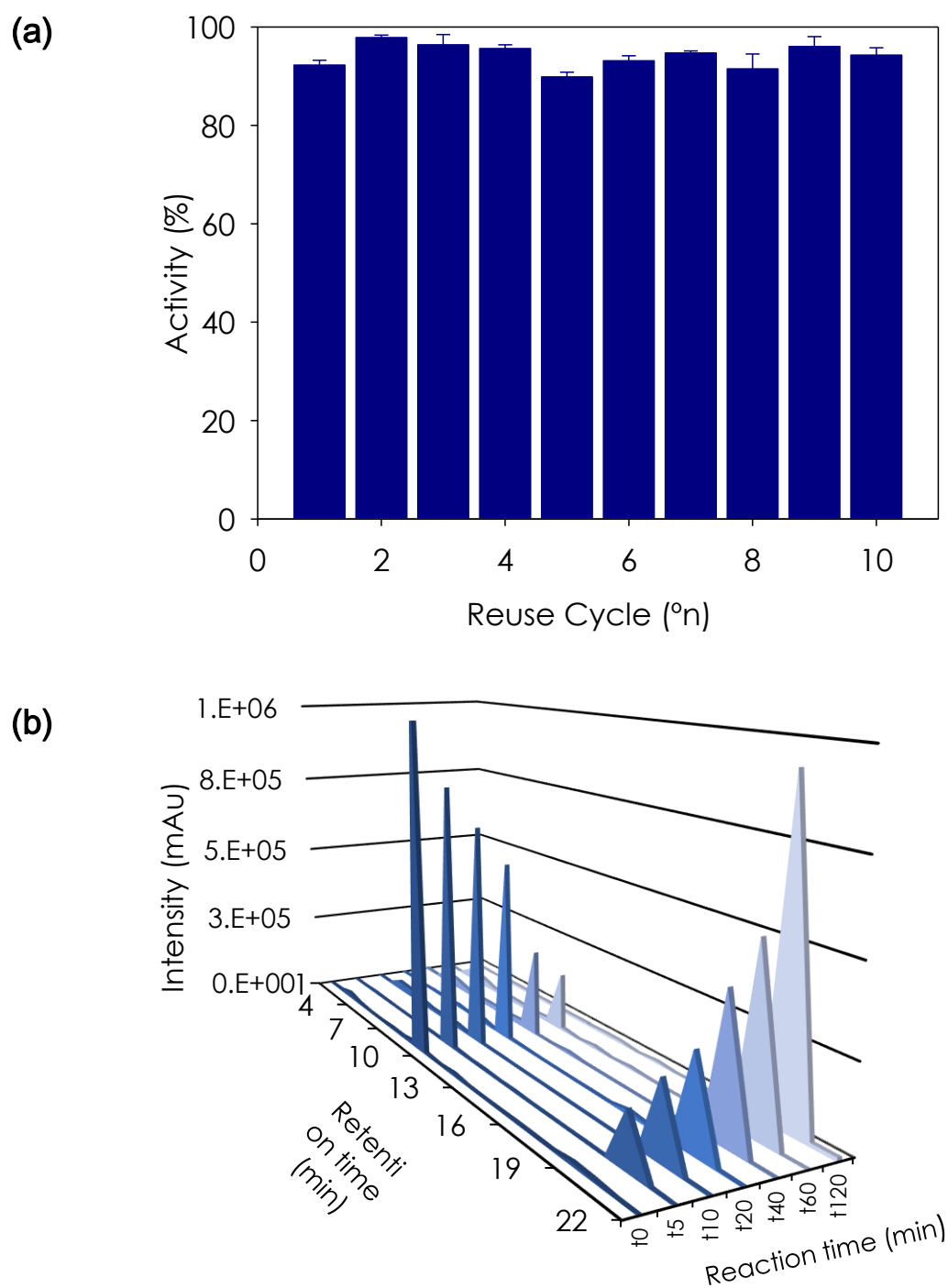


Fig 8: (a) Biocatalyst reusability in ten cycles of cinnabarinic acid production-recovery **(b)** HPLC chromatograms for cinnabarinic acid production (reuse cycle n° 5) by immobilized laccase, at different reaction times.

4 Conclusions

We have characterized the laccase mediated formation of cinnabarinic acid, a reagent with interesting pharmacological properties. The pH studies for the substrate and product revealed pH dependence for both of them and the substrate has a pKa value of 5.05. Also the product has a reactive intermediate that accumulates and gets oxidized to the final product cinnabarinic acid.

Anchoring laccase to the modified mesopores of the silica support by cross-linking them with a diepoxide may be considered economically viable and environmentally acceptable for oxidation reactions, such as the production of the valuable cinnabarinic acid (5.0 mg costs 87.80 € Sigma-Aldrich. Co. LLC.).

Supplementary Material

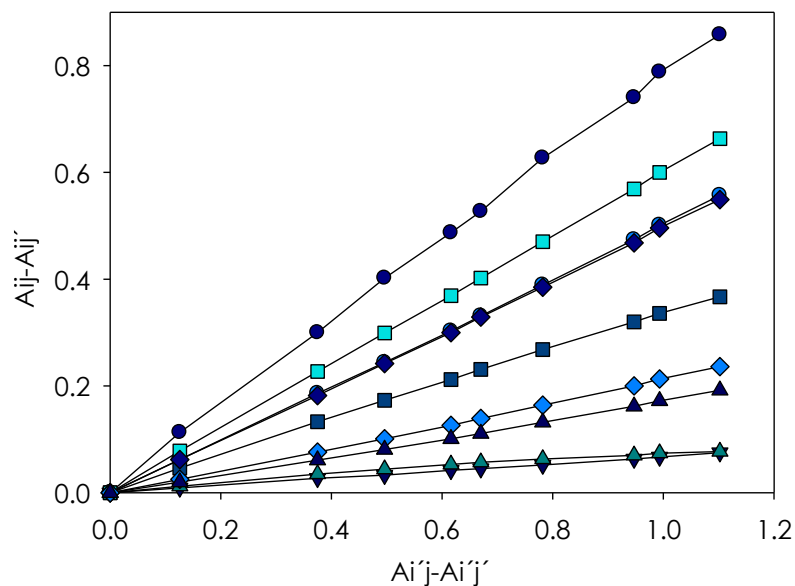


Fig 1s: Coleman graphical analysis for the repetitive spectra of **Fig 4 (a)**

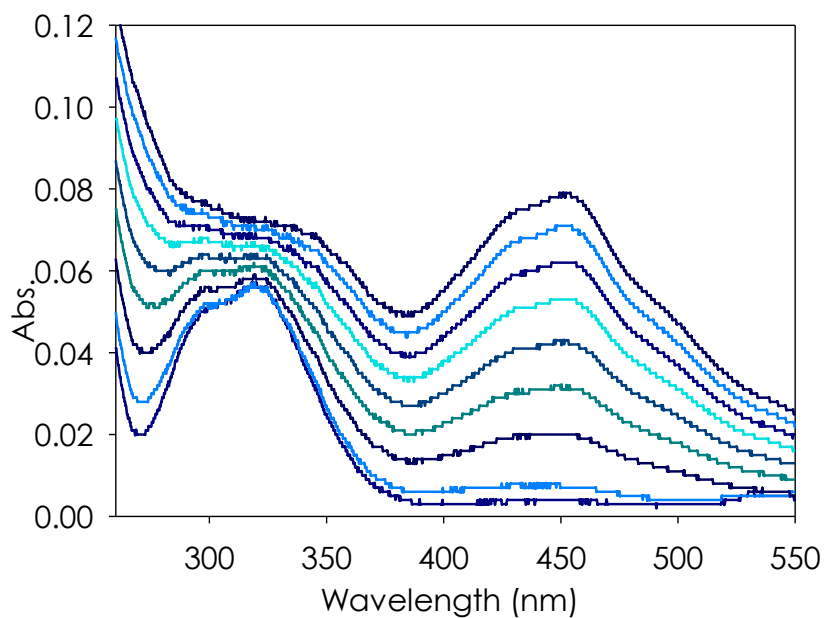


Fig 2s: Repetitive spectra of the laccase reaction in defect of substrate ($36 \mu\text{M}$), and low concentration of laccase ($0.4 \mu\text{g}$) pH 5.0 (50 mM sodium acetate) and 25°C . Spectra were recorded every 90 seconds.

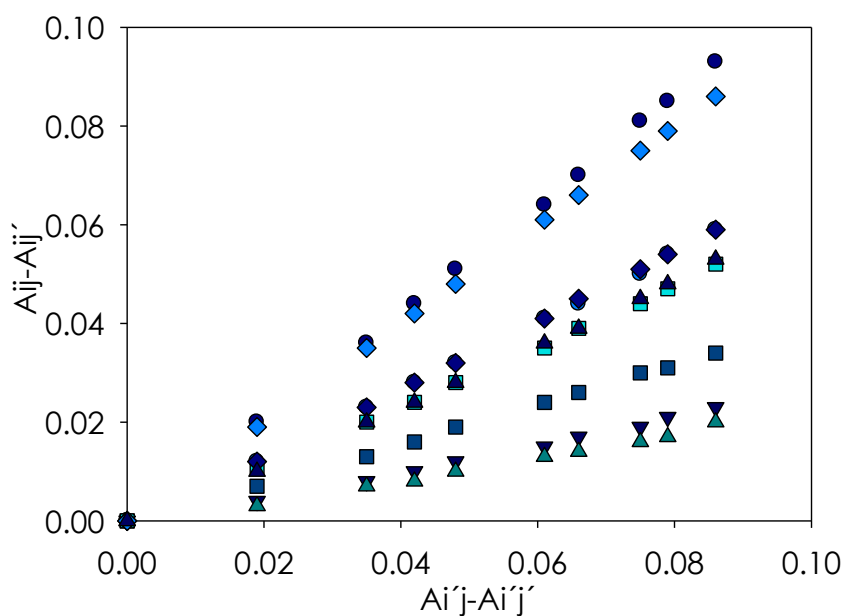


Fig 3s: Coleman graphical analysis for the repetitive spectra of **Fig 2s**.

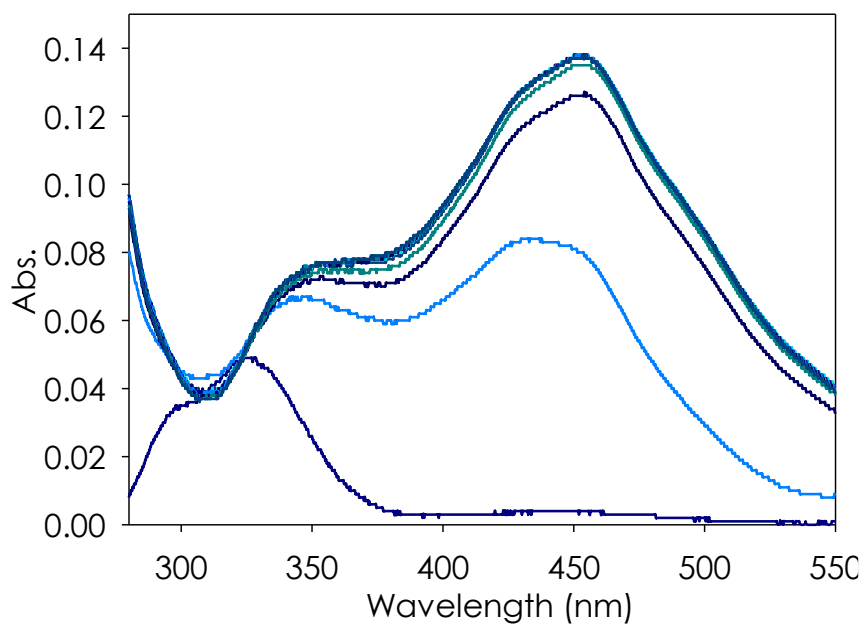


Fig 4s: Repetitive spectra of the laccase reaction in defect of substrate ($36 \mu\text{M}$), and high concentration of laccase ($10 \mu\text{g}$) pH 5.0 (50 mM sodium acetate) and 25°C . Spectra were recorded every 90 seconds.

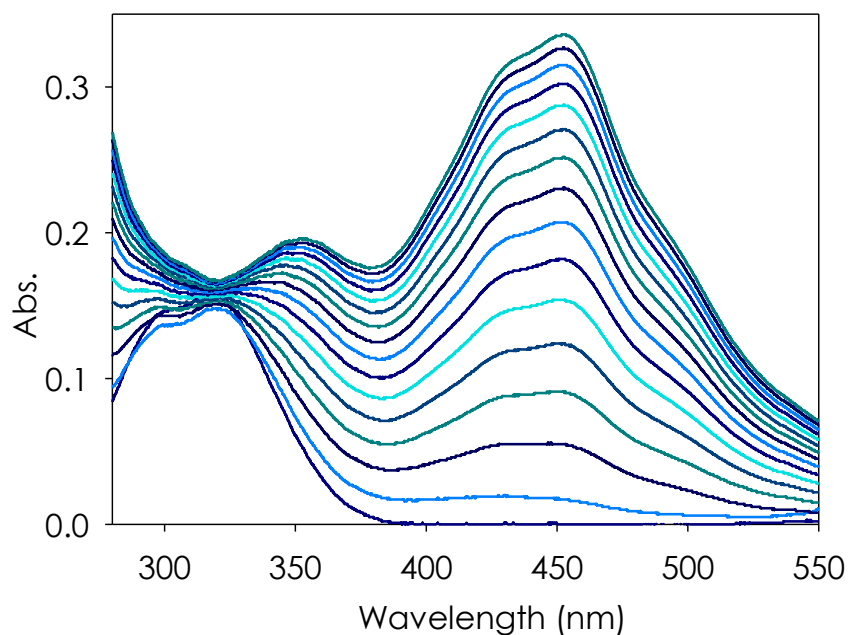


Fig 5s: Repetitive spectra of the laccase reaction in equimolar concentration of substrate ($120 \mu\text{M}$), and high concentration of laccase ($2 \mu\text{g}$) pH 5.0 (50 mM sodium acetate) and 25°C . Spectra were recorded every 90 seconds.

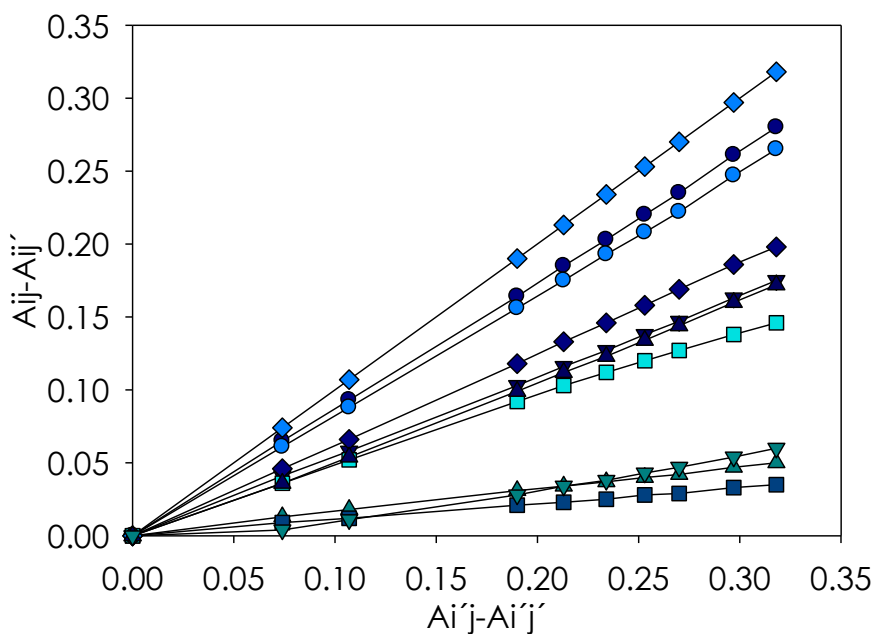


Fig 6s: Coleman graphical analysis for the repetitive spectra of **Fig 5s**.

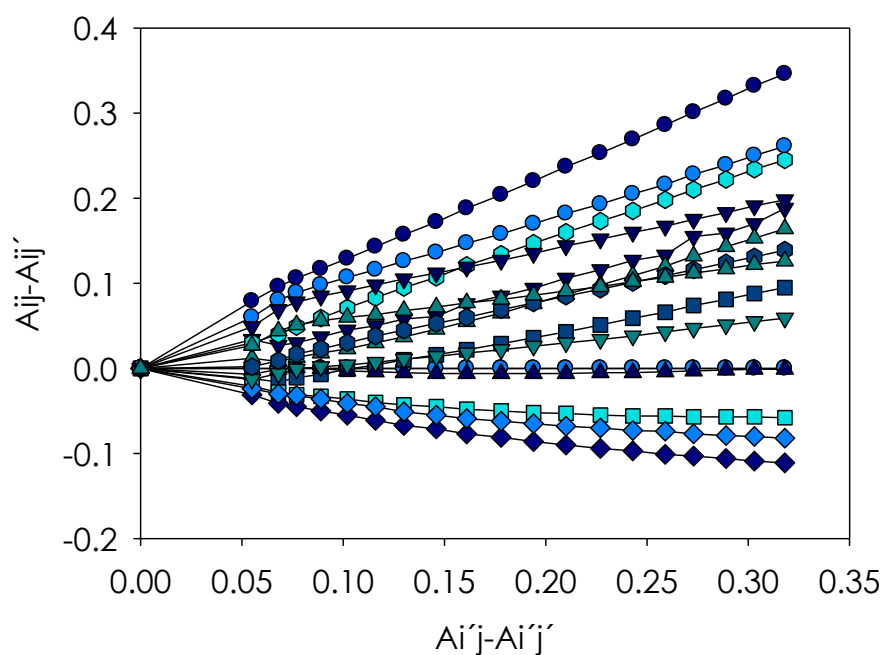


Fig 7s: Coleman graphical analysis for the repetitive spectra of **Fig 4 b**.

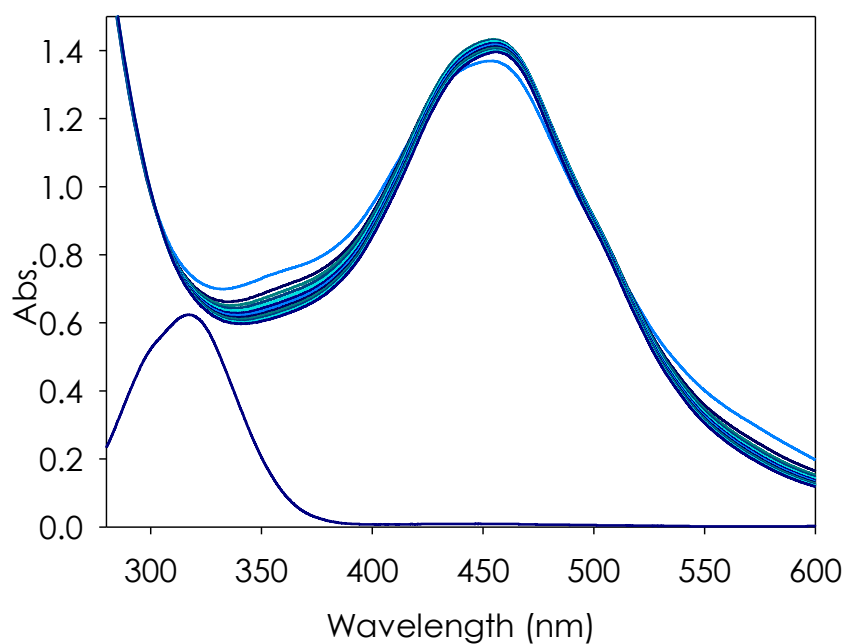


Fig 8s: Repetitive spectra of the periodate oxidation reaction in excess of sodium periodate (2 mM), and 200 μM concentration substrate, pH 5.0 (50 mM sodium acetate) and 25°C. Spectra were recorded every 90 seconds.

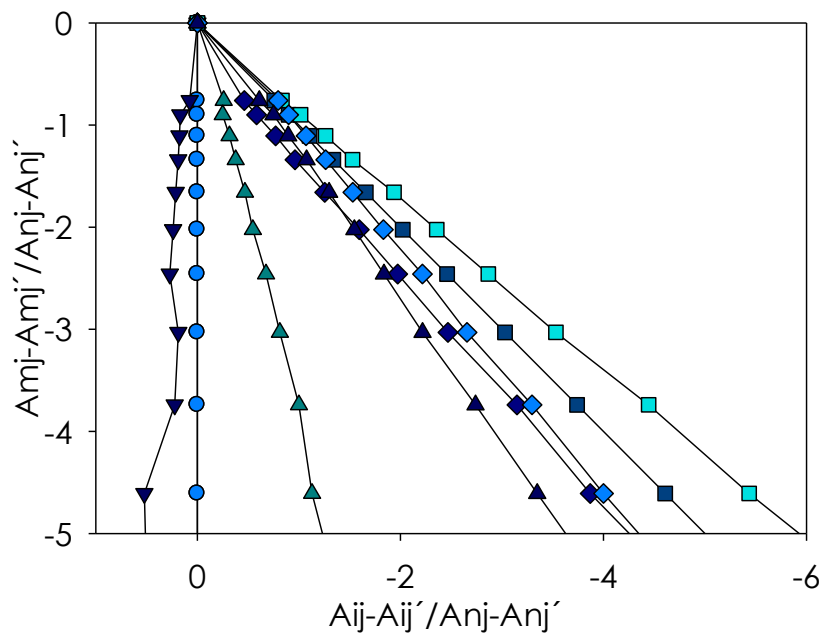


Fig 9s: Coleman graphical analysis for the repetitive spectra of **Fig 8s**.

5 References

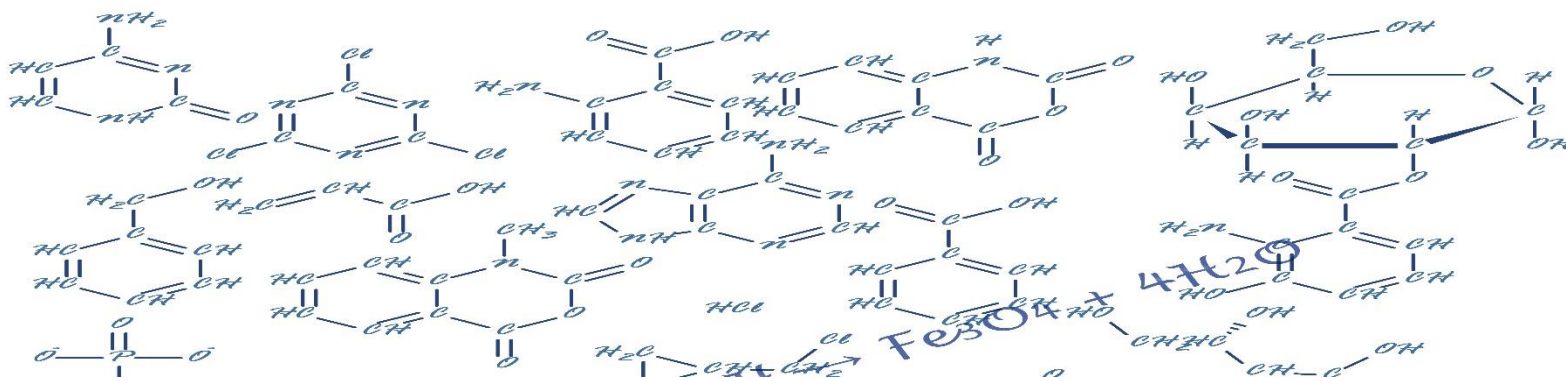
- Bruyneel, F. et al., 2008. Regioselective Synthesis of 3-Hydroxyorthanilic Acid and Its Biotransformation into a Novel Phenoxazinone Dye by Use of Laccase. *European Journal of Organic Chemistry*, 2008(1), pp.72–79.
- Cabana, H. et al., 2009. Immobilization of laccase from the white rot fungus *Coriolopsis polyzona* and use of the immobilized biocatalyst for the continuous elimination of endocrine disrupting chemicals. *Bioresource technology*, 100(14), pp.3447–3458.
- Cánovas, F.G. et al., 1982. The role of pH in the melanin biosynthesis pathway. *Journal of Biological Chemistry*, 257(15), pp.8738–8744.
- Chandrasekaran, J., 2001. Chemistry of colours. *Resonance*, 6(3), pp.66–75.
- Coleman, J.S., Varga, L.P. & Mastin, S.H., 1970. Graphical methods for determining the number of species in solution from spectrophotometric data. *Inorganic chemistry*, 9(5), pp.1015–1020.
- Dai, Y. et al., 2010. In situ encapsulation of laccase in microfibers by emulsion electrospinning: Preparation, characterization, and application. *Bioresource technology*, 101(23), pp.8942–8947.
- Darlington, L.G. et al., 2010. On the biological importance of the 3-hydroxyanthranilic acid: anthranilic acid ratio. *International Journal of Tryptophan Research*, 3, p.51.
- Eggert, C., 1997. Laccase-catalyzed formation of cinnabarinic acid is responsible for antibacterial activity of *Pycnoporus cinnabarinus*. *Microbiological research*, 152(3), pp.315–318.
- Fazio, F. et al., 2012. Cinnabarinic acid, an endogenous metabolite of the kynurenine pathway, activates type 4 metabotropic glutamate receptors. *Molecular pharmacology*, 81(5), pp.643–656.
- Fraenkel-Conrat, H., 1944. The action of 1, 2-epoxides on proteins. *Journal of biological chemistry*, 154(1), pp.227–238.

- Han, Y., Lee, S.S. & Ying, J.Y., 2006. Pressure-driven enzyme entrapment in siliceous mesocellular foam. *Chemistry of materials*, 18(3), pp.643–649.
- Hiramatsu, R. et al., 2008. Cinnabarinic acid generated from 3-hydroxyanthranilic acid strongly induces apoptosis in thymocytes through the generation of reactive oxygen species and the induction of caspase. *Journal of cellular biochemistry*, 103(1), pp.42–53.
- Iwahashi, H., 1999. 3-Hydroxyanthranilic acid-derived compounds formed through electrochemical oxidation. *Journal of Chromatography B: Biomedical Sciences and Applications*, 736(1), pp.237–245.
- Jimenez, M. et al., 1984. Chemical intermediates in dopamine oxidation by tyrosinase, and kinetic studies of the process. *Archives of biochemistry and biophysics*, 235(2), pp.438–448.
- Klibanov, A.M., 1997. Why are enzymes less active in organic solvents than in water? *Trends in biotechnology*, 15(3), pp.97–101.
- Lee, C.-H.H., Lin, T.-S.S. & Mou, C.-Y.Y., 2009. Mesoporous materials for encapsulating enzymes. *Nano Today*, 4(2), pp.165–179.
- Lloret, L. et al., 2012. Immobilisation of laccase on Eupergit supports and its application for the removal of endocrine disrupting chemicals in a packed-bed reactor. *Biodegradation*, 23(3), pp.373–386.
- Lorenzo, M. et al., 2005. Inhibition of laccase activity from *Trametes versicolor* by heavy metals and organic compounds. *Chemosphere*, 60(8), pp.1124–1128.
- Lu, L., Zhao, M. & Wang, Y., 2007. Immobilization of laccase by alginate–chitosan microcapsules and its use in dye decolorization. *World Journal of Microbiology and Biotechnology*, 23(2), pp.159–166.
- Mold, J.E., 2011. Use of cinnabarinic acid as a modulator of immune responses in autoimmune disorders. US Patent App. 13/579,893
- Rekuć, A. et al., 2010. Very stable silica-gel-bound laccase biocatalysts for the selective oxidation in continuous systems. *Bioresource technology*, 101(7), pp.2076–2083.

Chapter 6

- Roy, J.J. & Abraham, T.E., 2006. Preparation and characterization of cross-linked enzyme crystals of laccase. *Journal of Molecular Catalysis B: Enzymatic*, 38(1), pp.31–36.
- Schwarcz, R. et al., 2012. Kynurenines in the mammalian brain: when physiology meets pathology. *Nature Reviews Neuroscience*, 13(7), pp.465–477.
- Stone, T.W., Stoy, N. & Darlington, L.G., 2013. An expanding range of targets for kynurenine metabolites of tryptophan. *Trends in pharmacological sciences*, 34(2), pp.136–143.
- Tischer, W. & Wedekind, F., 1999. Immobilized enzymes: methods and applications. In *Biocatalysis-from discovery to application*. Springer, pp. 95–126. Available at: http://link.springer.com/chapter/10.1007/3-540-68116-7_4.
- Torres-pacheco, I., Octavio, L.C. & Guanajuato, C., 2006. No Title. , 661(2), pp.323–340.
- Zhu, Y. et al., 2007. Immobilization of *Trametes versicolor* laccase on magnetically separable mesoporous silica spheres. *Chemistry of Materials*, 19(26), pp.6408–6413.

Chapter 7



Functionalization of magnetic particles
by means of nucleophilic substitution
of organic halides

Abstract

In this work magnetic nanoparticles (9-12 nm) are directly functionalized by nucleophilic substitution with organic halides. These halides are organic compounds containing one or more halogens groups, like epichlorohydrin, 1, 3, 5 trichloro 2, 4, 6 triazine, cyanogen bromide and chloride, p-toluenesulfonyl chloride or other organic halides able to react with hydroxyl groups. This method improves the general functionalization procedure, which includes a polymer coating over the magnetic particles then the functionalization process. Polymeric layers tend to act as inert shield that hinder the particle magnetism, our direct modification in the particle surface avoids this phenomenon, don't increase the size of the particles and is economically sustainable.

The obtained magnetic nanoparticles, functionalized with this method, have reactive groups as cyanate, chloride, epoxy or tosyl groups available to react with biological molecules, as proteins, enzymes, nucleic acids, or antibodies and bind them covalently to the magnetic surface. They could be also chemically modified, by adding reactive groups such as organic amines the particles nature could be changed, making them more hydrophilic (positive or negative charged) or hydrophobic.

Keywords: magnetic nanoparticles, surface modification, organic halide, substitution reaction, SN1

1 Introduction

Magnetic particles are applied in many areas in industry due to their advantages over other materials. They are applied in the manufacture of structural materials, such as ceramics and composites, to improve their mechanical performance. They are also used in sealed vacuum systems, damping systems, speakers, magnetic sensors, actuators, catalysis, metal recovery and water purification, inductors and antennas. The particles are in the form of films, powder or fluid dispersed in aerosol form.

One of the first applications of the magnetic particles in the field of biomedicine was biotechnology about the 70s, these particles were used as carriers of enzymes, in separation processes, purification and analysis of proteins, in biocatalysis and bioprocesses (Magnogel, Dynabeads and Estapor). Already in the 80s they began to be used as contrast agents in magnetic resonance imaging (MRI). Recently have been described a variety of utilities such as directed drug delivery, immunoassays, molecular biology, nucleic acid purification (DNA, RNA), cell separation, hyperthermia therapy, and others (Tartaj et al. 2003). Industrially this particles are used in sealed vacuum systems, damping systems, magnetic sensors, actuators, metal recovery and water purification, also in magnetic printing, magnetic inks, and lubrication. These applications are based on their high specific surface, in its ability to cross biological barriers, ion adsorption capacity, and mainly in its unique magnetic, optical, and electrical properties, etc.

Several techniques have been developed to prepare suspensions of these magnetic particles with organic and inorganic coatings technology and biomedical applications (Laurent et al. 2008). The first requirement in the manufacture of such suspensions is to achieve stability of the same. A second point is to incorporate one or more features (chemical active groups) on the surface of the particles. To achieve both is usual to coat the particles with an organic or inorganic material which acts as

protection for the particles, improves their stability, avoid the oxidation and can incorporate functional groups on their surface. There are several strategies for coating particles that can be classified according to the type of compound used in the coating: polymer coatings, silica coatings or molecular interaction with small molecules as oleic acid or phosphine (Lu et al. 2007).

The functionalization of the particles, i.e. anchoring active agents is performed on the surface of the stabilizing layer once coated particle. This strategy entails some drawbacks as purification processes and limitations on the reaction conditions to prevent aggregation or degradation of the stabilizing layer or to add functionality itself. The functionalization can be accomplished by electrostatic adsorption (Wang et al. 2009) but is preferably carried out by covalent bonding through a spacer which connects the coating surface of the particle with the active agent (Georgelin et al. 2010). A versatile method is based on specific binding between streptavidin and biotin, although it is costly and requires the use of biotinylated active agents.

In this work a novel method to stabilize and functionalize magnetic nanoparticles is proposed. We theorized that the hydroxyl groups on the magnetic nanoparticles surface could be used as organics alcohols and are available to react with organic halides via nucleophilic substitution. The nucleophilic substitution reaction of organic halides with alcohols is well known and industrially used as the use of epichlorohydrin for the treatment of natural and synthetic fibers (Stallmann 1934; Paul 1938; Granacher 1936) the preparation of dyes with triazine chloride (Wieners 1935). Also immobilizing proteins by cyanogen bromide activated supports (Axen et al. 1972). The particles reacts with the organic halides and are activated to attach biological (enzymes, nucleic acids, and antibodies) or chemical molecules on its surface.

2 Materials and Methods

2.1 Materials

Iron chloride (III) hexahydrate, iron sulfate (II) heptahydrate, epichlorohydrin (99 %), 2, 4, 6-Trichloro-1, 3, 5-triazine (99 %), Amano Lipase from *Pseudomonas fluorescens* (> 20000 U/ g), DMSO (> 99%) were purchased from Sigma Aldrich, acetone HPLC grade was obtained from Fisher Scientific.

2.2 Methods

2.2.1 Magnetic Nanoparticles preparation

Magnetic particles were synthesized as reported Kim et al (Kim et al. 2001), with slightly modifications, 13.51 g of $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ were dissolved in 25 mL of distilled water, 6.95 g of $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ were dissolved in 25 mL of 0.5 M HCl. The solutions were mixed and dropped on 150 mL of NaOH 5 M, with high stirring (1500-2000 rpm). A black precipitated with magnetic properties was immediately formed. Once all the iron solution is dropped, the mixture was neutralized (pH 7.0) with HCl 5 M. Using a neodymium magnet the supernatant was removed and the particles were washed three times with 100 mL of deionized water. The synthesized magnetic particles had a mean size of 9-12 nm.

2.2.2 Magnetic nanoparticles Epichlorohydrin Surface functionalization

Five grams of magnetic nanoparticles were dissolved in 50 mL of deionized water and sonicated ten minutes in a water bath sonicator (Branson). Epichlorohydrin (12 mL) was dissolved in 8 mL of DMSO and added to the magnetic nanoparticles suspension, the mixture was sonicated other ten minutes. Then the pH was adjusted to 12.0 with NaOH 0.1 M, and the mixture was left under mild stirring overnight at room temperature. Then the activated nanoparticles were washed with acetone once

and with water twice. Activated magnetic nanoparticles were stored in 40 % DMSO at 4 °C.

2.2.3 Magnetic nanoparticles 1, 3, 5 Trichloro 2, 4, 6 triazine Surface functionalization

One gram of magnetic nanoparticles were dissolved in 50 mL of deionized water and sonicated ten minutes in a water bath sonicator (Branson). 1, 3, 5 trichloro 2, 4, 6 triazine (1 g) was dissolved in 50 mL of acetonitrile and added to the magnetic nanoparticles suspension, the mixture was sonicated other ten minutes. Then the pH was adjusted to 12.0 with NaOH 0.1 M, and the mixture was left under mild stirring overnight at 50 °C. Then the activated nanoparticles were washed with acetone once and with water twice. Activated magnetic nanoparticles were stored in acetone at 4°C.

2.2.4 TEM characterization

Nanoparticles were characterized with a transmission electron microscopy Philips Tecnai (FEI corporate, Oregon) at acceleration voltage of 120 kv with negative stain. One microliter of the sample was fixed in a cooper support film grid and dyed with uranyl acetate solution, then visualized with the electron microscopy.

2.2.5 Titration of epoxy groups in the surface

Epoxides in surface were determined with sodium thiosulfate, 500 mg of the sample were incubated with 3.0 mL sodium thiosulfate (1.3 M) overnight at room temperature. Then diluted with 7.0 mL of deionized water and titrated with 0.1 M of HCl till pH 7.0 using phenolphthalein (1 % w/v in ethanol) as indicator. Epoxy groups were calculated following Eq. 1:

$$N_{epoxy} = \frac{0.1(V_1 - V_0)}{1000 M} = \frac{epoxy\ mol}{support\ weight} \quad (\text{Eq. 1})$$

V_1 is the volume of HCl used in the samples (mL).

V_0 is the volume of HCl used in the blank assay (mL).

M is the support weight (mg).

2.2.6 Lipase immobilization with epoxy magnetic nanoparticles

Lipase covalent immobilization on the magnetic support was made as follows: thirty milligrams of magnetic particles functionalized with epichlorohydrin were re-suspended in 2 mL of potassium phosphate buffer (50 mM, pH 7.0), on the other hand 30 mg of Lipase from *Pseudomonas fluorescens* was dissolved in 4 mL of potassium phosphate buffer (50 mM, pH 7.0) to which 500 μ L of PEG 600 was added. The enzyme solution was added to the particles suspension and allowed to react in a water bath at 30 ° C for 6 hours. Then the particles were washed once with ethanol and twice with potassium phosphate buffer (50 mM, pH 7.0).

The enzyme not bound to the support was quantified by the Bradford method (Bradford 1976).

Lipase activity was measured with *p*-nitrophenyl acetate as substrate (Hernández-García et al. 2014). In brief, 5 μ L of *p*-nitrophenylacetate (1 mg/mL dissolved in acetone), was added to 975 μ L of citrate buffer (50 mM, pH 6.5) in a 1 mL spectrophotometric cuvette, the reaction was started by adding 20 μ L of free lipase (1mg/mL), supernatant sample or immobilized lipase. The release of *p*-nitrophenol was measured at 400 nm at 30 °C.

2.2.7 Amine surface modification

Amine incorporation to the magnetic particles was made as follows: 100 mg of magnetic particles functionalized with triazine chloride were re-suspended in 1 mL of distilled water, to the particle suspension 1 mL of ethylene diamine was added and the mixture was incubated 12 hours with moderate stirring at 50 ° C. Particles were

washed twice with ethanol and twice with water. Amines in surface were determined with the naphthoquinone method.

2.2.8 Amine determination with Naphthoquinone sulfonate

Surface amines were determined following the naphthoquinone method (Muszalska et al. 2015) with some modifications, 100 μL of the particles suspension were dissolved in 350 μL of buffer (66 % NaOH 0.2 M, 25 % KCl 0.2 M, adjusted with KCl 0.2 M to pH 13.0), then 50 μL of naphthoquinone sulfonate (0.5 % w/v in water) was added. The mixture was incubated 15 minutes at 60 $^{\circ}\text{C}$, then diluted with 500 μL and measured at 483 nm.

Calibration curve was prepared with the same procedure using 100 μl of ethanolamine solutions with concentrations between 0 and 2 mM.

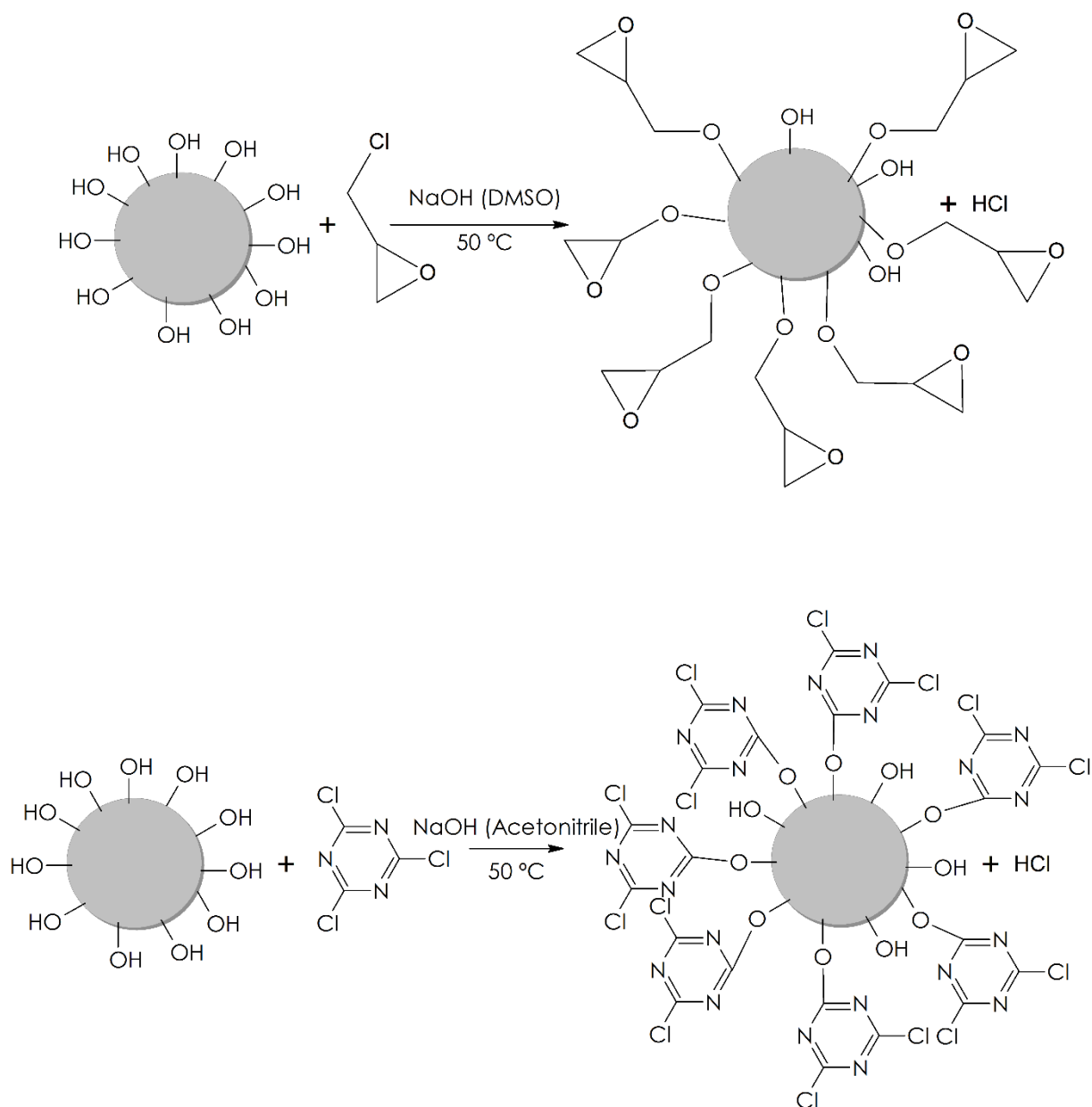
3 Results and Discussion

3.1 Organic halides magnetic particles direct functionalization

The schematic representation of the functionalization procedure can be seen in Scheme 1, we supposed that if the iron oxides were reactive then they would have similar behavior as semi-metal oxides like silicon oxide or aluminum oxides. These oxides can react with epichlorohydrin (Hsiue et al. 1999) to give functionalized materials. In effect as we theorized the hydroxyl groups of the magnetic nanoparticle are reactive enough to be attacked with the organic halide and produce the desired substitution reaction. The reactivity of the hydroxyl groups of magnetite in difference with other iron oxides had been described (Joseph et al. 1999).

As can be seen the reaction produces hydrochloric acid in the proposed cases, so the reaction pH has to be corrected during the reaction course, as the kinetic is slower in pH lower than 9.0, also the temperature is maintained in 50 °C to accelerate the reaction.

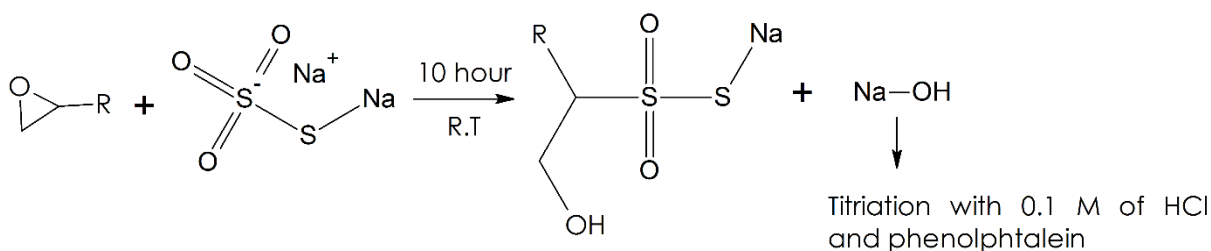
The TEM images of the particles showed that the procedure did not increase the size of the magnetic nanoparticles (9-12 nm).



Scheme 1: Schematic overview of the magnetic nanoparticles functionalization with organic halides.

3.2 Titration of surface epoxides

Reaction scheme for epoxide titration is presented in Scheme 2, sodium thiosulfate attacks the epoxy ring and is incorporated to the structure. The reaction releases sodium hydroxide that is titrated against hydrochloric acid using phenolphthalein as indicator. Phenolphthalein is pink during the titration reaction (pH above 7.0), and the color disappears, marking the end of the reaction (pH 7.0).



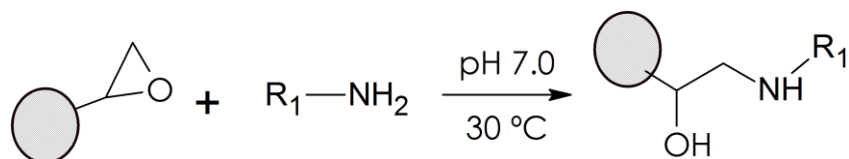
Scheme 2: Determination of epoxy groups with sodium thiosulfate method.

With the determination of oxirane groups with thiosulfate 3.2 mmol of oxirane groups per gram of magnetic nanoparticles were obtained. Commercial magnetic particles with the same functionalization had 250 $\mu\text{mol/g}$ (BcMag™ Epoxy-Activated Magnetic Beads, Bioclone Inc), 100-200 $\mu\text{mol/g}$ (Dynabeads® M-270 Epoxy, Invitrogen). However, our produced magnetic particles had an average size of 12 nm, while the commercial particles had a mean size of 1 μm (Bioclone) and 2.8 μm (Invitrogen).

3.3 Lipase immobilization with epoxy magnetic nanoparticles

The produced particles were used as a carrier to immobilize enzymes. The chosen enzyme was lipase (E.C. 3.1.1.3) from *Pseudomonas fluorescens*, as depicted the Scheme 3. The oxirane groups reacts with the aminoacids lateral chains, most probably with amino groups to attach the enzyme at neutral pH, and 30 °C to increase the

reaction rate. PEG600 was added as active site protector and to maintain the open conformation of the lipase (Schrag et al. 1997; Mateo et al. 2007).

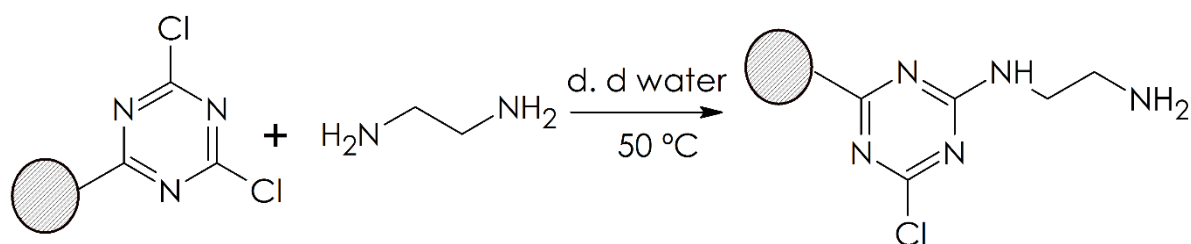


Scheme 3: Epoxy reaction with primary amines.

From the 30 mg of lipase added in the assay only 0.25 mg were unbound and present in the supernatant. Therefore our epoxy derivatized particles immobilized around 991 mg of lipase per gram of magnetic particles. The activity yield was 73.63 % for the hydrolysis of *p*-nitrophenyl acetate. Lipase from *Candida rugosa* was immobilized in γ -Fe₂O₃ particles with an average size of 20 nm, 55.6 μ g of lipase was immobilized per milligram of particles (Dyal et al. 2003). Yong reported a lipase from *Candida rugosa* immobilized on magnetic microspheres via epoxy groups, the prepared biocatalyst retained 64.2 % activity (Yong et al. 2008).

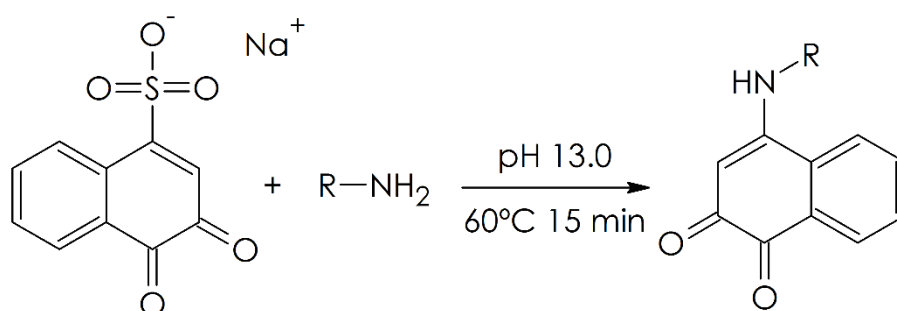
3.4 Amine surface modification

For the production of hydrophilic magnetic particles, the trichloro triazine activated particles were reacted with diethylene diamine in excess, the reaction was depicted in Scheme 4. The amines were bound to the triazine ring while hydrochloric acid was released, the reaction was heated to 50 °C to increase the reaction rate and the incorporation of amines to the magnetic particles.



Scheme 4: Triazine reaction with ethylene diamine.

The resulting particles were more hydrophilic and did not aggregated in water at slightly acidic pH, while naked magnetic nanoparticles tended to aggregate in water. Amine groups on the particles surface were measured with the naphthoquinone sulfonate method (Muszalska et al. 2015), showed in Scheme 5, using ethanolamine to prepare the calibration curve (Fig 1). The results showed 2.21 mmol amines per gram of nanoparticles.



Scheme 5: Naphthoquinone titration method for primary amines.

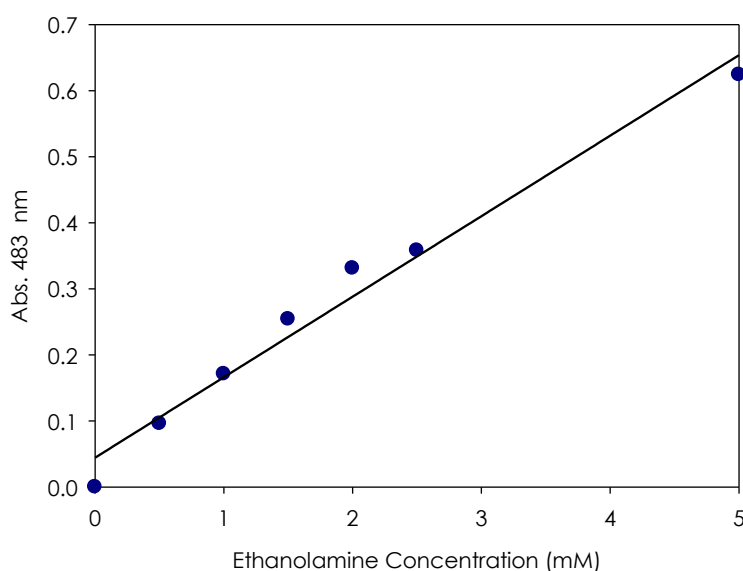


Fig 1: Calibration curve of ethanolamine with naphthoquinone sulfonate.

4 Conclusions

A fast and simple method to functionalize and activate magnetic nanoparticles was proposed. In this work the reactivity of the –OH groups in the surface of magnetite magnetic nanoparticles was used to incorporate functional motifs on its surface. Using organic halides, as epichlorohydrin or trichlorotriazine, in basic pH a reaction type SN1 takes place. The organic part of the halide was attached to the magnetic particle releasing hydrochloric acid. The obtained particles had active groups than could be reacted again, epichlorohydrin magnetic particles had 3.2 mmol of oxirane per gram of particles. While commercial particles had around 100-250 μmol of oxirane per gram of particles. Note than our functionalized particles were smaller (12 nm) than the commercial ones (1-2.8 μm).

This new functionalization method maintain the particles nanosize and its magnetic properties (superparamagnetism or low magnetic remanence), while protecting them from oxidation. The method reduced the production cost of the functionalized particles by reducing the fabrication and purification steps, also using organic halides that are low cost reagents used in industrial processes as cotton dying.

Epichlorohydrin functionalized magnetic particles were used as support to immobilize enzymes. The support can attach around 991 mg of enzyme per gram of particles, and the enzymes maintained a 73.63 % activity.

5 References

Axen, R.E.A. V, Porath, J.O. & Ernback, E.S., 1972. Method of binding water-soluble proteins and water-soluble peptides to water-insoluble polymers using cyanogen halide. US Patent 3,645,852

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1–2), pp.248–254.

Dyal, A. et al., 2003. Activity of *Candida rugosa* lipase immobilized on γ -Fe₂O₃ magnetic nanoparticles. *Journal of the American Chemical Society*, 125(7), pp.1684–1685.

Georgelin, T. et al., 2010. Nanoparticle-Mediated Delivery of Bleomycin. *Angewandte Chemie International Edition*, 49(47), pp.8897–8901.

Granacher, C., 1936. Ethereal cellulose derivatives and process of making the same. US Patent 2,054,746

Hernández-García, S., García-García, M.I. & García-Carmona, F., 2014. Purification, Immobilization and Characterization of Lipase Isoenzyme from *Aspergillus niger* with C8 Magnetic Particles. *Advances in Bioscience and Biotechnology*, 5(7), p.633.

Hsiue, G.-H., Wang, W.-J. & Chang, F.-C., 1999. Synthesis, characterization, thermal and flame-retardant properties of silicon-based epoxy resins. *Journal of applied polymer science*, 73(7), pp.1231–1238.

Joseph, Y. et al., 1999. Adsorption of water on FeO (111) and Fe₃O₄ (111): identification of active sites for dissociation. *Chemical physics letters*, 314(3), pp.195–202.

Kim, D.K. et al., 2001. Synthesis and characterization of surfactant-coated superparamagnetic monodispersed iron oxide nanoparticles. *Journal of Magnetism and Magnetic Materials*, 225(1), pp.30–36.

Laurent, S. et al., 2008. Magnetic iron oxide nanoparticles: synthesis, stabilization, vectorization, physicochemical characterizations, and biological applications. *Chemical reviews*, 108(6), pp.2064–2110.

Lu, A., Salabas, E.L. & Schüth, F., 2007. Magnetic nanoparticles: synthesis, protection, functionalization, and application. *Angewandte Chemie International Edition*, 46(8), pp.1222–1244.

Mateo, C. et al., 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and microbial technology*, 40(6), pp.1451–1463.

Muszalska, I. et al., 2015. 1, 2-Naphthoquinone-4-sulfonic acid sodium salt as a reagent for spectrophotometric determination of rimantadine and memantine. *Journal of Analytical Chemistry*, 70(3), pp.320–327.

Paul, S., 1938. Process of increasing the fastness of dyeings. US Patent 2,131,121

Schrag, J.D. et al., 1997. The open conformation of a *Pseudomonas lipase*. *Structure*, 5(2), pp.187–202.

Stallmann, O., 1934. Vat dyestuff paste. US Patent 1,977,252

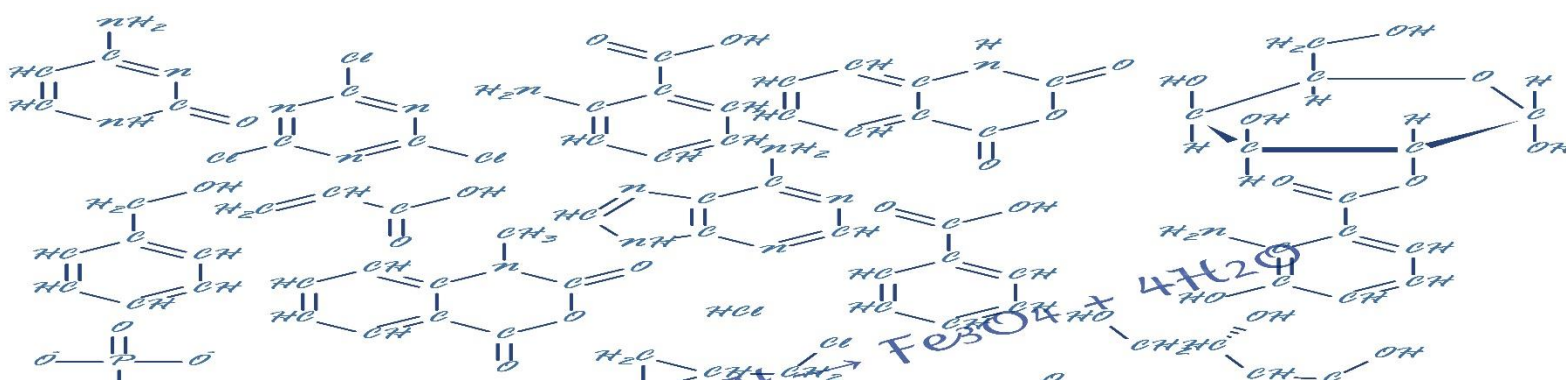
Tartaj, P. et al., 2003. The preparation of magnetic nanoparticles for applications in biomedicine. *Journal of Physics D: Applied Physics*, 36(13), p.R182.

Wang, X. et al., 2009. Control of aggregate size of polyethyleneimine-coated magnetic nanoparticles for magnetofection. *Nano Research*, 2(5), pp.365–372.

Wieners, F., 1935. Process of preparing vat dyestuffs of the anthraquinone triazine series. US Patent 1,994,602

Yong, Y. et al., 2008. Characterization of *Candida rugosa* lipase immobilized onto magnetic microspheres with hydrophilicity. *Process Biochemistry*, 43(11), pp.1179–1185.

Chapter 8



Magnetic Nanometric Lipase for
solvent free Ester Synthesis.

Abstract

A sustainable method to obtain a seminatural benzyl acetate in a solvent free system is proposed. Using a magnetic nanometric (15 nm) lipase biocatalyst, produced with our patented method. Firstly the magnetic particles were prepared by coprecipitation method and functionalized with organic halides (epichlorohydrin and cyanogen bromide) that reacts with the hydroxyl groups on the particle surface. Then the enzyme was covalently attached to the particles surface obtaining a nanosize biocatalyst. The biocatalyst was used for the synthesis of benzyl acetate in a solvent free reaction.

Solvent free benzyl acetate synthesis was studied, using the prepared biocatalyst or free lipase. The substrate molar rate (vinyl acetate and benzyl alcohol), the amount of water, and the temperature were optimized, also the kinetic parameters were determined. In the end with the nanometric biocatalyst a 90 % conversion of benzyl alcohol to benzyl acetate can be attained in 10 hours with a maximal amount of benzyl acetate produced of 300 g per liter. The magnetic catalyst allowed and easy separation and recovery of the enzyme using a neodymium magnet, obtaining an enzyme-free final product. The biocatalyst can also be reused up to ten times without loss of performance.

Keywords: magnetic nanoparticles, lipase, solvent free synthesis, esterification, biocatalyst, non-conventional media

1 Introduction

Magnetic particles have drawn many attentions in the last few decades, in specially the magnetic nanoparticles because their unique properties like the superparamagnetic behavior. The superparamagnetism is a property that only occurs when the size of the particle is below 50 nm. Then each nanoparticle becomes a single magnetic domain with a fast response to applied magnetic fields with negligible remanence (residual magnetism) and coercivity (the field required to bring the magnetization to zero). These features make superparamagnetic nanoparticles very attractive for a broad range of applications, including magnetic fluids, catalysis, biotechnology/biomedicine, magnetic resonance imaging, data storage, and environmental remediation. However such smaller particles tend to aggregate in bigger cluster to reduce the energy associated with the high surface area to volume ratio of the nanosized particles. Also naked metallic nanoparticles are chemically highly active, and are easily oxidized in air, resulting generally in loss of magnetism and dispersibility. Usually the aggregation and the environmental oxidation could be avoided coating the nanoparticles with a polymeric inert material such as silica, the siliceous coating prevent the oxidation, minimized the aggregation and facilitates the functionalization of the particles for immobilization purposes (Lu et al. 2007). In this work we present a new –patented (Garcia-Carmona et al. 2015)- methodology to protect and functiontionalize the nanometric magnetic particles without polymeric coerture. The new method consist in an in-situ reaction of the hydroxyl groups in the magnetite surface with an organic halide, attaching covalently the group to the magnetic surface.

The magnetic particles were modiflicated with epichlorohydrin and cyanogen bromide, in order to have reactive (cyane and epoxy) groups to covalently attach industrially relevant enzymes such as lipase (Singh et al. 2013). The magnetic lipase

was tested in a solvent free synthesis reaction, to produce the short chain ester, benzyl acetate.

Benzyl acetate a short chain ester present in jasmine, ylang ylang and tobira plants (Fig 1). It have a characteristic pleasant fruity odor and it is very used in cosmetic, fragrance, food and pharmaceutical formulates. The natural extracted compound is not a viable source of obtaining due its minority presence in the vegetal sources (Welsh et al. 1989). Organic synthesis of the compound is a cheaper method of obtaining, however the organic synthesis involves the use of unhealthy chemical compounds used as catalyst like sulfuric acid, heavy metals (tungsten, molybdenum, or titanium), organic compounds (pirrolydones) or ionic liquids. These methods not only present the problem of the unhealthy catalyst, most of them have low yield, as H_2SO_4 with 65.8 % yield or iron salts that yields a 67.1 %, are very expensive, use hard working conditions (more than 100 °C, ultrasonic frequencies, long reaction times, organic solvents) and generates large amount of waste water (Cong 2012). A biotechnology approach could be a more economic, healthy and successful way for the synthesis of benzyl acetate. The use of a magnetic lipase in a solvent free system (one of the substrates is used as solvent), is cheaper because the catalyst is cheap and could be easily removed and reused (Chen et al. 2013), avoids the use of organic solvents, omit the generation of waste water, avoids the presence of heavy metals, ionic liquids in the final product, the working conditions are mild, and the consumer view of this semisynthetic product is much better than the organic synthesized one (Garlapati & Banerjee 2013). The consumer have a better approach to products that can be labeled as “natural” in comparison with the ones labeled “synthetic”, this preference involves more demand of natural labeled products and in consequence an increase in the price of natural labeled products (Welsh et al. 1989).



Fig 1: Tobira (*Pittosporum tobira*) left and Ylang-Ylang (*Cananga odorata*) flowers right

In this work we proposed a new technique to modify magnetic particles without disturbing the size or the magnetic properties of the particles. We attached lipase to the surface of the particles and tested the biocatalyst in a solvent free media to produce benzyl acetate. The process was optimized and the most important variables studied.

2 Materials and Methods

2.1 Materials

Amano Lipase from *Pseudomonas fluorescens* (≥ 20000 U/g), *p*-nitrophenyl butyrate, tributyrin, ethylene glycol were purchased from Sigma Aldrich. All the materials to prepare the magnetic particles were purchase from sigma Aldrich. Benzyl alcohol, benzyl acetate and vinyl acetate were from TCI. Rest of materials were also from Sigma Aldrich of HPLC grade.

2.2 Methods

2.2.1 Magnetic Nanoparticles preparation

Magnetic particles were synthesized as reported Kim et al (Kim et al. 2001), with slightly modifications, 13.51 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were dissolved in 25 mL of distilled water, 6.95 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were dissolved in 25 mL of 0.5 M HCl. The solutions were mixed and dropped on 150 mL of NaOH 5 M, with high stirring (1500-2000 rpm). A black precipitated with magnetic properties was immediately formed. Once all the iron solution was dropped, the mixture was neutralized (pH 7.0) with HCl 5 M. Using a neodymium magnet the supernatant was removed and the particles were washed three times with 100 mL of deionized water. The synthesized magnetic particles had a mean size of 9-12 nm.

2.2.2 Magnetic Nanoparticles surface activation

Surface activation of the nanoparticles was made using our patented method (Garcia-Carmona et al. 2015). For the epichlorohydrin activated particles 5 g of magnetic nanoparticles were dissolved in 50 mL of deionized water and sonicated ten minutes in a water bath sonicator (Branson). Epichlorohydrin (12 mL) was dissolved in 8 mL of DMSO and added to the magnetic nanoparticles suspension, the mixture

was sonicated other ten minutes. Then the pH was adjusted to 12.0 with NaOH 0.1 M, and the mixture was left under mild stirring overnight at room temperature. Then the activated nanoparticles were washed with acetone once and with water twice. Activated magnetic nanoparticles were stored in 40 % DMSO at 4 °C.

For the activated cyane nanoparticles, one hundred milligrams of nanoparticles were activated in 1 mL of sodium borate buffer (0.2 M pH 8.5). Particles were collected with a magnet and the supernatant removed.

Cyanogen bromide was freshly prepared dissolving 0.5 g in 1 mL of acetonitrile, and added to the magnetic particles, then mixed with vortex and left to stand in ice for ten minutes. Then the particles were collected with a magnet, the supernatant discarded and washed twice with cold PBS (phosphate saline buffer). The particles were used immediately to lipase immobilization.

Nanoparticles were characterized with a transmission electron microscopy Philips Tecnai (FEI corporate, Oregon) at acceleration voltage of 120 kV with negative stain. One microliter of the sample was fixed in a cooper support film grid and dyed with uranyl acetate solution, then visualized with the electron microscopy.

2.2.3 Lipase immobilization procedures

The magnetic epichlorohydrin or cyane activated nanoparticles (100 mg) were washed with acetone once and twice with potassium phosphate buffer (50 mM, pH 7.0), and left in 1 mL of the same buffer. Lipase (100 mg) was also dissolved in the same buffer with 0.25 % of tributyrin. The solutions were mixed and left to react with continuous stirring 3 hours at 30 °C.

Then supernatant was extracted and keep for further analysis, the lipase-magnetic particles were washed three times with tris/acetate buffer (50 mM pH 6.5), diluted to 10 mg/mL with the same buffer and stored at 4°C.

Protein present in the supernatants was measured with bicinchoninic acid method (Smith et al. 1985).

2.2.4 Lipase activity test

Free and immobilized lipase activity was measured with 4-nitrophenyl ester substrates, following our improved method. Briefly, *p*-nitrophenyl butyrate was dissolved in ethylene glycol in a waterbath sonicator at 500 mM, then adjusted to 25 mM with tris/acetate 50 mM pH 7.5, being the final concentration of ethylene glycol 5 % In a 1 mL spectrophotometer cuvette 850 μ L of Tris/acetate buffer 50 mM pH 7.5 with 5 % of ethylene glycol and 100 μ L of the prepared substrate were added, reaction was started by adding 20 μ L of free lipase (1 mg/mL) of immobilized lipase (1 mg support/mL). The formation of the yellow compound *p*-nitrophenol was measured at 400 nm in a Shimadzu spectrophotometer with a temperature controller set at 30 °C.

2.2.5 Benzyl acetate Synthesis

Benzyl acetate was produced in a solvent free system using vinyl acetate as the acyl donor and solvent and benzyl alcohol as acceptor. A minimal optimized amount of water was added to the system. Several variables were studied to optimize the conversion of benzyl alcohol to benzyl acetate.

The general assay contains substrate mix (1:1), 100 μ L of water (2.75 M) in form of potassium phosphate buffer (20 mM, pH 7.0) and 20 mg of lipase free or immobilized in the magnetic support, being the final volume 2 mL. The assays were maintained at 30 °C, with continuous orbital shaking (250 rpm). Ten microliters aliquots were taken a fixed times, diluted in methanol (990 μ L), centrifuged 10 minutes, 10000 \times g at 4°C to remove protein contaminants and stored at 4 °C for further analysis. Blank assays were prepared in the same way, but contained no enzyme.

2.2.5.1 Vinyl acetate and benzyl alcohol molar relation

The relationship between substrates towards lipase was analyzed testing different molar rates of benzyl alcohol/vinyl acetate. The reaction volume was fixed 2 mL, the water content 100 μ L (2.75 M) in form of potassium phosphate buffer (20 mM, pH 7.0) and lipase (20 mg). The reaction time was 48 hours and aliquots (10 μ L) were taken every hour for the first six hours, then every twelve hours.

2.2.5.2 Temperature profile

The lipase behavior towards the temperature was studied and optimized, testing a temperature range between 25-50 $^{\circ}$ C. The samples contained 20 mg of lipase, 2 mL of substrate mix (0.25:1 benzyl alcohol: vinyl acetate) and 2.75 M water in form of buffer phosphate (20 mM, pH 7.0). The assays were kept under continuous shaking (250 rpm), for six hours.

2.2.5.3 Minimal water amount

For the water content experiments, lipase (20 mg) was firstly dehydrated with several absolute ethanol washes. Then the required amount of water (in form of buffer phosphate 20 mM, pH 7.0), was pipetted and mixed with vortex. The reaction then was started by adding 2 mL of substrate mixture (0.25:1 benzyl alcohol: vinyl acetate), the reaction was left to progress at 30 $^{\circ}$ C with orbital shaking (250 rpm) for six hours. Aliquots (10 μ L) were taken every hour, diluted in methanol and stored at 4 $^{\circ}$ C for further analyses.

2.2.5.4 Kinetic parameters

Michaelis-Menten equation for a bisubstrate reaction as

Benzyl alcohol + vinyl acetate \rightarrow Benzyl acetate

A+B \rightarrow C

Can be written as

$$V_0 = \frac{V_{MAX} [A][B]}{K_{MA}[B] + K_{MB}[A] + [A][B]} \quad \text{(Eq. 1)}$$

In our case A (vinyl acetate) \gg B (benzyl alcohol) so

$$\xrightarrow{[A] \rightarrow \infty} \text{Then } \frac{K_{MA}}{[A]} \rightarrow 0$$

Simplifying the equation results in

$$V_0 = \frac{V_{MAX}}{\frac{K_{MBapp}}{[B]} + 1} \quad \text{(Eq.2)}$$

Reordering the equation, the result is a Michaelis-Menten equation function of [B] (benzyl alcohol).

$$V_0 = \frac{V_{MAX} [B]}{K_{MBapp} + [B]} \quad \text{(Eq.3)}$$

Maintaining concentration of vinyl acetate constant and tenfold higher than benzyl alcohol, was possible to obtain the Michaelis-Menten parameters for benzyl alcohol. Using 20 mg of immobilized lipase, 0.66 M of water in form of phosphate buffer (20 mM, pH 7.0). Reactions were placed under orbital shaking at 30 °C. Benzyl alcohol concentration was varied between 0.01-1 M in order to obtain the kinetic constant.

2.2.6 Biocatalyst recycle

To assess biocatalyst performance ten cycles of reuse were performed. For this 100 mg of the magnetic lipase were prepared in the optimum conditions. The reaction medium (10 mL) contained the substrates benzyl alcohol and vinyl acetate (0.25:1), 0.66 M of water in form of phosphate buffer and 100 mg of immobilized lipase. The reaction was placed in an orbital shaker with the temperature fixed at 30 °C. The reaction was stopped after ten hours, the immobilized lipase was collected using a neodymium

magnet and the reaction medium was discarded. Then the immobilized lipase was supplied with fresh reaction medium (10 mL).

2.2.7 HPLC analysis of Benzyl acetate

An easy HPLC method was proposed for the detection of Benzyl alcohol, vinyl alcohol and benzyl acetate in a HPLC Shimadzu prominence modular HPLC (Shimadzu Corporation, Tokyo Japan). A C18 Kromasil HPLC column (250 mm × 4.6 mm) was used with a linear gradient of methanol 70% at 0.5 mL/min.

Calibration curves were prepared for the substrates and product, retention time for benzyl alcohol (4.2 min at 250 nm), vinyl acetate (4.75 min at 230 nm) and benzyl acetate (7.25 min at 250 nm).

3 Results and Discussion

3.1 Magnetic nanoparticles preparation and functionalization

The Fig 2 is the electron images of the epichlorohydrin modified particles, as could be seen the modified particles retain its nanometric size approximately 13.2 nm with a standard deviation of 1.03 nm.

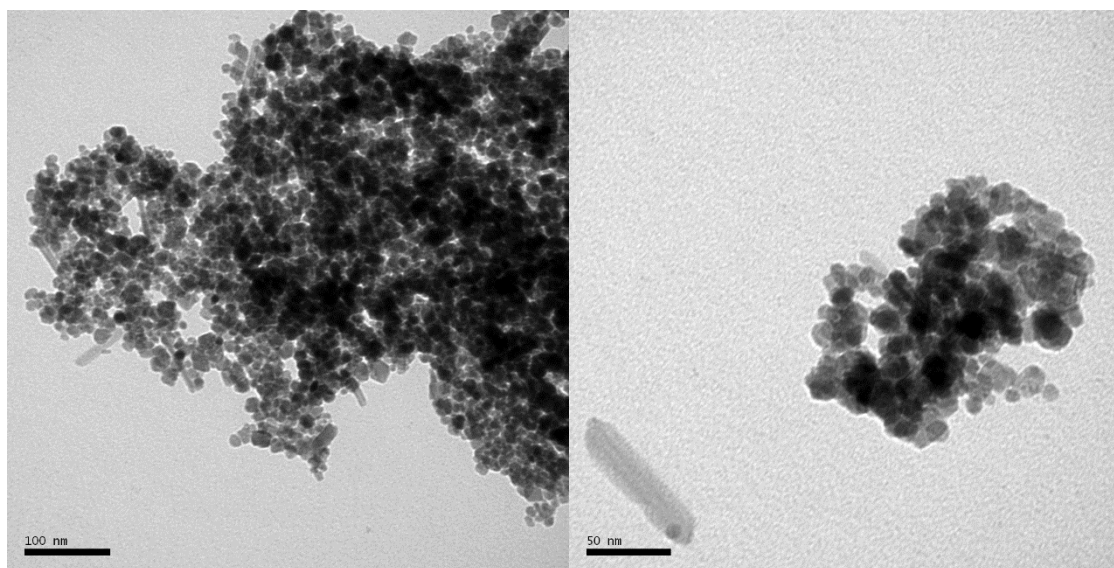


Fig 2: TEM images of the modified magnetic nanoparticles.

3.2 Lipase immobilization

Cyanide derivatized support showed less capacity as support for lipase immobilization than the epichlorohydrine one, as the cyanide supports admitted less enzyme mass (0.01 mg attached per mg supplied) than the epoxide one (1 mg attached per mg supplied) using 10 mg of support. The recovered activity was also less for the cyane support (60 % of activity) whilst the epoxy support had a 110 % of activity, showing lipase superactivation phenomenon produced by the hydrophobic support as reported Fernandez-Lafuente (Fernandez-Lafuente et al. 1998).

The optimization of enzyme mass for 10 mg of epoxy support was illustrated in Fig 3. By supplying 10 mg of lipase per 10 mg of support almost all the enzyme is attached to the support, however the activity yield was lower than the support with less enzyme attached. With an initial enzyme mass of 1 mg, 1 mg was immobilized and the immobilized enzyme had 110% of activity. Adding tributyrin to the reaction mix, allowed the enzyme to be immobilized in the open lid conformation form, the active form (Fernandez-Lafuente et al. 1998) improving its activity.

The biocatalyst preparation selected to be used in the benzyl acetate synthesis was 10 mg of support charged with 1 mg of lipase.

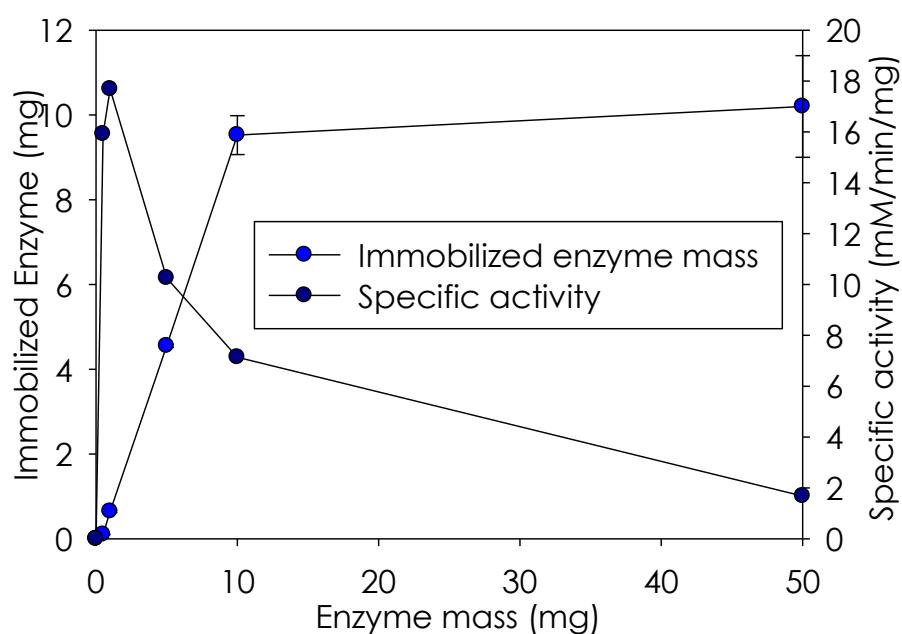
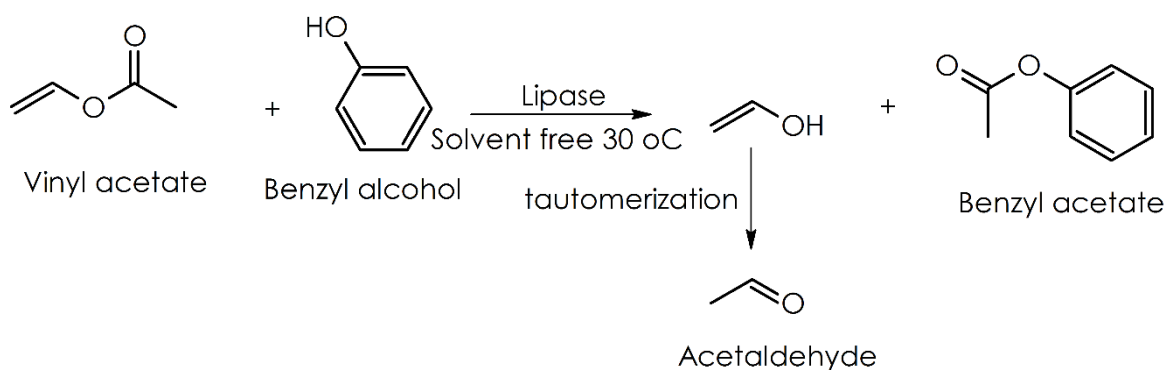
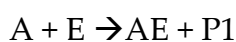


Fig 3: Relationship between the enzyme mass supply to be immobilized with the final mass immobilized (●) in magnetic epichlorohydrin support (10 mg) at pH 7 (20 mM potassium phosphate buffer 0.025% tributyrin), and its specific hydrolytic activity (●), measured with p-nitrophenyl butyrate at pH 7.5 (50 mM tris/acetate 2% ethylene glycol) at 30°C.

3.3 Benzyl acetate synthesis

The synthesis of benzyl acetate from benzyl alcohol was illustrated in Scheme 1. Using vinyl acetate as acyl donor, the products obtained were benzyl acetate and vinyl alcohol that was tautomerized to acetaldehyde. It can be described as a Ping-Pong Bi Bi mechanism (Paiva et al. 2000) without inhibition. The enzyme (E) forms a complex with the acyl donor (vinyl acetate) (A), and the first product, vinyl alcohol is released and transformed in acetaldehyde. Then the second substrate (benzyl alcohol) (B) binds to the acetylated enzyme and forms the complex acyl-enzyme-alcohol. This complex will dissociate in enzyme (E) and final product benzyl acetate.



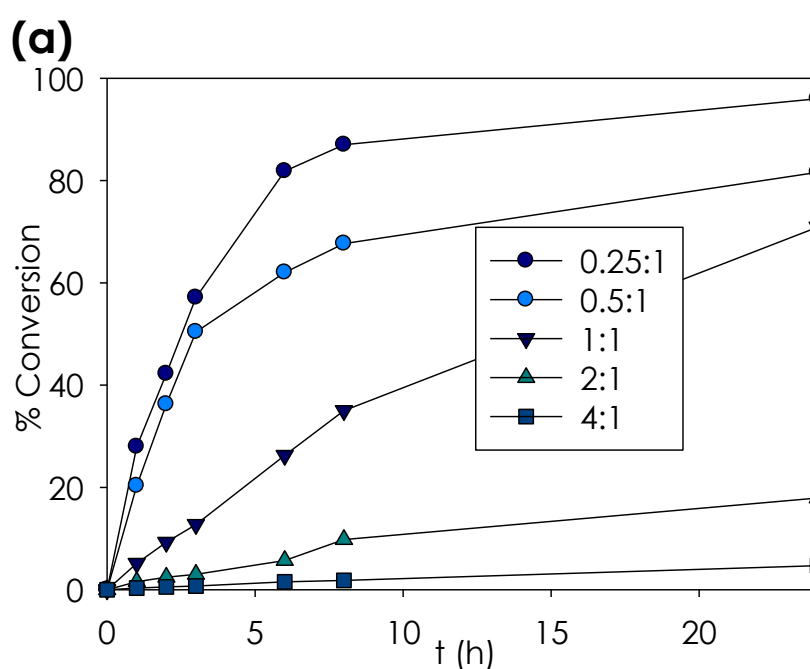
Scheme 1: Benzyl alcohol synthesis scheme.

3.3.1 Vinyl acetate and benzyl alcohol molar rate

The effect of substrates molar rate (benzyl alcohol: vinyl acetate) towards the conversion was shown in Fig 4 (a) for the immobilized lipase and in Fig 4 (b) for the free lipase. As can be seen the highest yields were obtained when the acyl donor was in excess relative to the alcohol. A conversion of 90 % for benzyl alcohol was yielded in 6 hours and it reached a 95 % in 24 hours using immobilized lipase whilst the free lipase yields an 80 % in twenty four hours. Vinyl acetate was chosen as acyl donor,

because the reaction cannot be reversed as vinyl alcohol was irreversibly tautomerized to acetaldehyde that was continuously eliminated in gas form. Before choosing vinyl acetate as acyl donor acetic acid and acetic anhydride were tested as well, while using vinyl acetate the conversion of benzyl alcohol reached 60 % in 3 hours without optimization, the others only yielded 12 % for acetic acid and 30 % for acetic anhydride. Unlike acetic acid, vinyl acetate did not change the pH of the medium during the reaction (Romero et al. 2005), and also was more affordable than the other acyl donors.

The low yields when the acyl donor was in defect indicated the necessity of the enzyme to be acylated before binding to the nucleophile substrate and be transformed (ping-pong Bi Bi mechanism). Remarkably the free lipase works better in defect of acyl donor than the immobilized lipase.



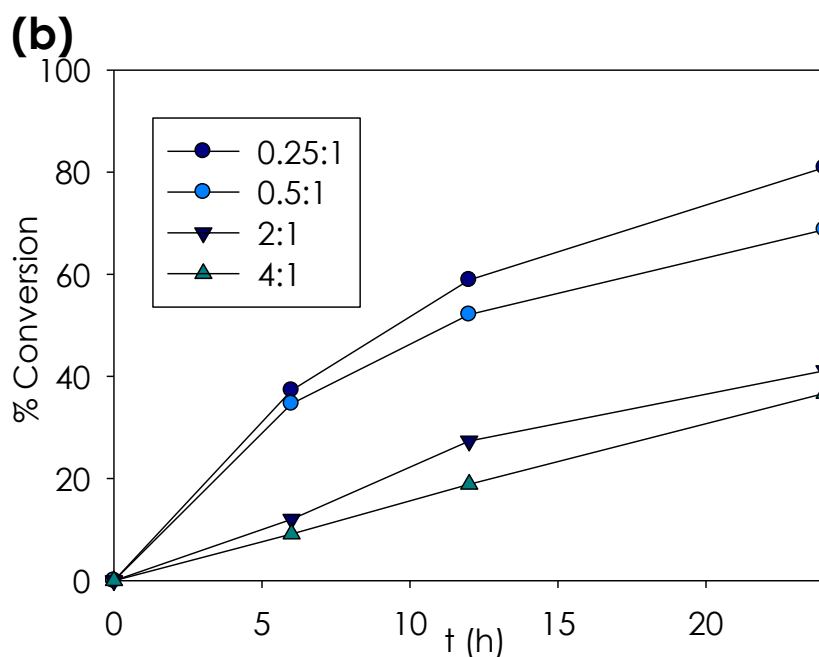


Fig 4: Effect of substrate molar rate for benzyl acetate synthesis using 20 mg of immobilized lipase **(a)** or 20 mg of free lipase **(b)**, with 100 μL of phosphate buffer 20 mM pH 7.0 at 30 $^{\circ}\text{C}$ under continuous shaking (250 rpm).

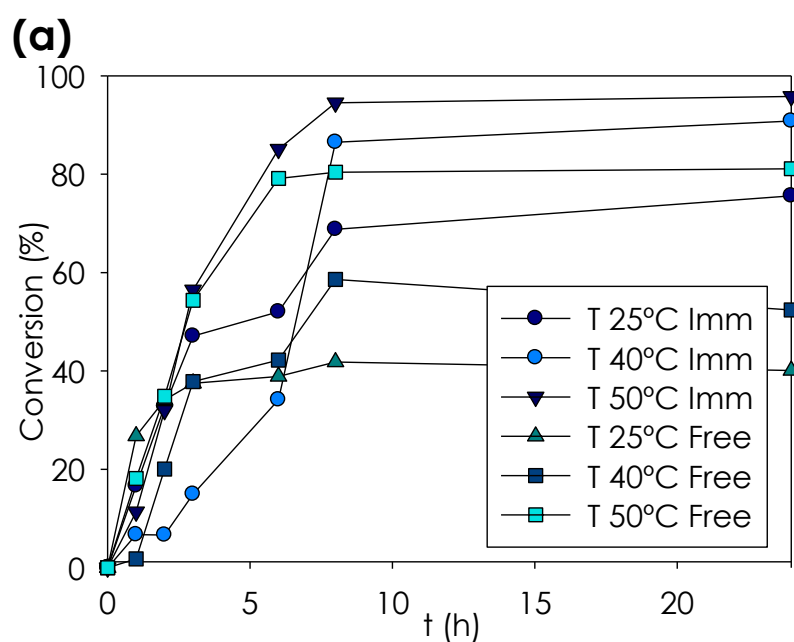
3.3.2 Temperature profile

The temperature profile of the lipase for the synthesis reaction showed minimal differences between 30, 40 and 50 $^{\circ}\text{C}$, however the initial rate was higher at 50 $^{\circ}\text{C}$ as can be seen in Fig 5 (a). Nevertheless for the free lipase the temperature increase the production of benzyl alcohol up to 80 % at 50 $^{\circ}\text{C}$. Despite this increase in production the temperature was maintained at 30 $^{\circ}\text{C}$ to avoid vinyl acetate loss by evaporation.

3.3.3 Minimal water amount

The water content was an influential parameter both in the conversion and in the initial rate of the esterification reaction. Lipases require a minimum of amount of water to catalyze reactions in organic solvents (Kvittingen et al. 1992). Normally, in esterification reactions that use acids as acyl donor the amount of water will be varied in the reaction course. Nevertheless with vinyl acetate the amount of water will be

constant in the reaction. In Fig 5 (b) the influence of water concentration towards reaction initial rate and final conversion is illustrated for free and immobilized lipase. The optimal water concentration for free and immobilized lipase was 0.66 M, this value gave the best conversion and the higher reaction rates. As can be seen in the Fig 5 (b), while the immobilized lipase worked with largest amounts of water, the free lipase was almost inactivated.



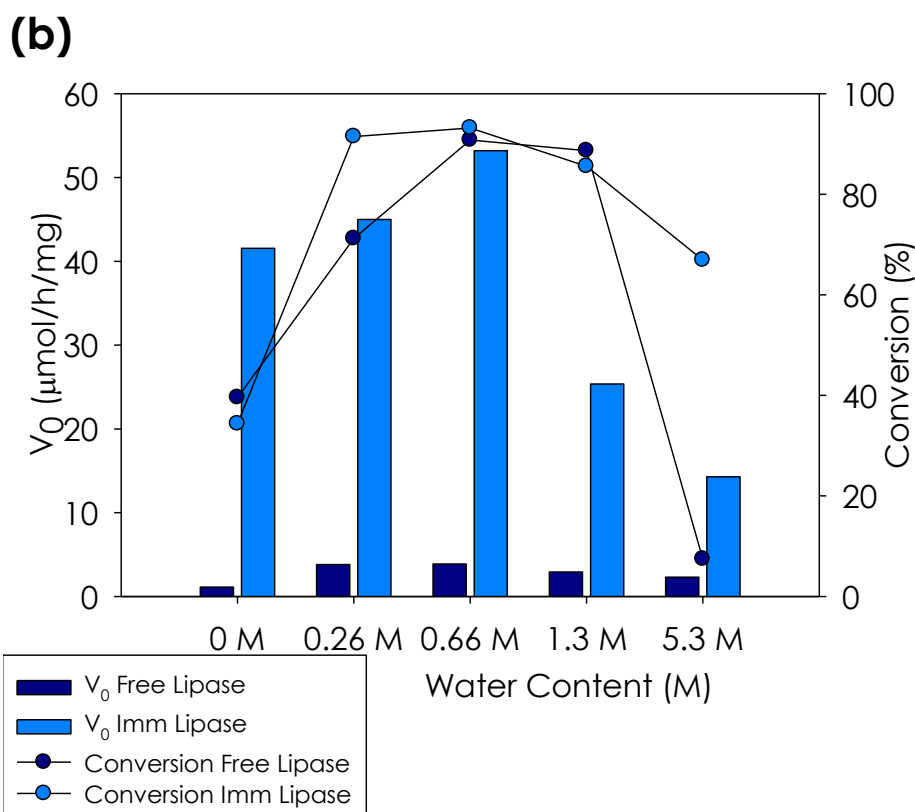


Fig 5: (a) Temperature effect towards ester synthesis for the free and the immobilized lipase (20 mg), using a 0.25: 1 molar ratio of benzyl alcohol: vinyl acetate, with 100 μL of phosphate buffer 20 mM pH 7.0 under continuous shaking (250 rpm). **(b)** Water amount effect on ester synthesis (conversion and initial rate) for the free and the immobilized lipase (20 mg), using a 0.25: 1 molar ratio of benzyl alcohol: vinyl acetate, at 30 $^{\circ}\text{C}$ under continuous shaking (250 rpm).

3.3.4 Kinetic study

The parameters obtained for the Michaelis-Menten adjust were V_{MAX} 24.12 nmol min^{-1} and a kinetic constant K_{MBapp} 0.89 M. Larger K_{M} have been associated with solvent free and organic solvent reactions (Liu et al. 2015; Chowdary & Prapulla 2005), the situation is less pronounced in non-polar solvents as hexane (Chowdary & Prapulla 2005), however the solvent free system is more attractive, as the purification process of the obtained product is easier and economically sustainable.

3.4 Biocatalyst reuse

The immobilized lipase showed good reusability as depicted Fig 6, several cycles of reuse can be attained without loss of performance, the final conversion was almost the same in the ten cycles.

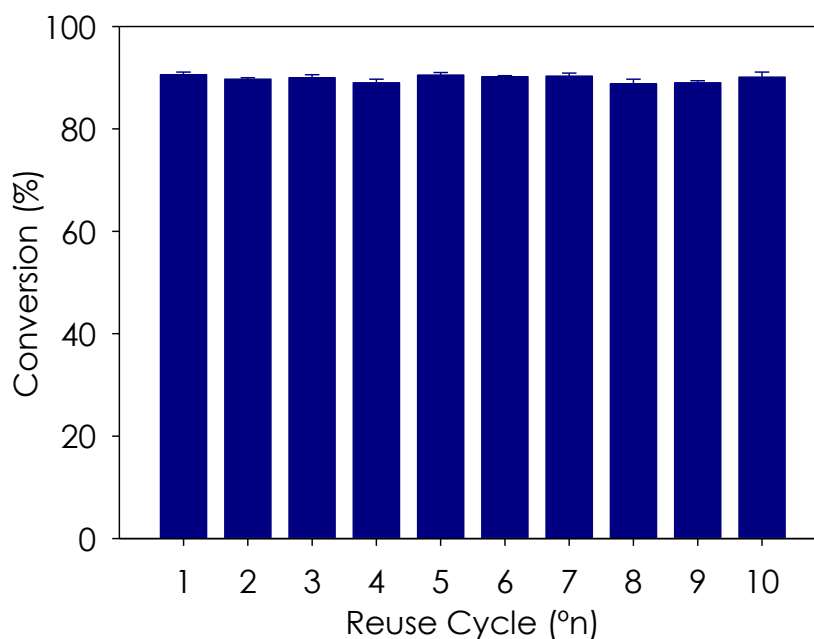


Fig 6: Biocatalyst reuse, the reaction medium (10 mL) contained 0.66 M of water (using phosphate buffer), benzyl alcohol: vinyl acetate (0.25:1) and 100 mg of lipase. Reaction was left to proceed for ten hours, at 30 °C under orbital shaking.

In comparison with the bibliography, *Rhizopus oryzae* lipase immobilized on Celite 545 was used to synthesize butyl acetate (pineapple flavour) in a solvent free reaction, the conversion was low (25 %) and after the third reuse cycle the biocatalyst started to lose performance (Salah et al. 2007). Lipase (Lipozyme-TL) from *Thermomyces lanuginose* was immobilized in magnetic particles and used in the transesterification of soy bean oil with methanol, the biocatalyst lost half the activity by the fifth cycle (Xie & Ma 2009).

4 Conclusions

A sustainable magnetic nanometric lipase is produced, using surface activate magnetic nanoparticles with epichlorohydrin. The support retains a 110% of activity indication lipase superactivation by the support. The biocatalyst is used to optimize the solvent free biocatalytic production of benzyl acetate. A natural compound presented in flowers and fruits and used in pharmacology, perfume and food industries. The relation of substrates, temperature and water amount is optimized to give a 90 % in ten hours and an amount of 300 g of benzyl alcohol per liter of reaction. Also the biocatalyst can be reused up to ten cycles without loss of performance.

5 References

- Chen, Y.Y. et al., 2013. Covalent immobilization of *Bacillus licheniformis* γ -glutamyl transpeptidase on aldehyde-functionalized magnetic nanoparticles. *International Journal of Molecular Sciences*, 14(3), pp.4613–4628.
- Chowdary, G. V & Prapulla, S.G., 2005. Kinetic study on lipase-catalyzed esterification in organic solvents. *Indian journal of chemistry section B*, 44(11), p.2322.
- Cong, Y., 2012. An overview on synthetic methods of n-butyl acetate. *European Chemical Bulletin*, 1(8), pp.336–337.
- Fernandez-Lafuente, R. et al., 1998. Immobilization of lipases by selective adsorption on hydrophobic supports. *Chemistry and Physics of Lipids*, 93(1), pp.185–197.
- Garcia-Carmona, F., Hernandez-Garcia, S. & Garcia-Garcia, M.I., 2015. Funcionalización de partículas magnéticas mediante sustitución nucleofílica de haluros orgánicos.
- Available at: <https://www.google.es/patents/WO2015092106A1?cl=es>.
- Garlapati, V.K. & Banerjee, R., 2013. Solvent-Free Synthesis of Flavour Esters through Immobilized Lipase Mediated Transesterification. *Enzyme research*, 2013, p.367410.
- Kim, D.K. et al., 2001. Synthesis and characterization of surfactant-coated superparamagnetic monodispersed iron oxide nanoparticles. *Journal of Magnetism and Magnetic Materials*, 225(1), pp.30–36.
- Kvittingen, L. et al., 1992. Use of salt hydrates to buffer optimal water level during lipase catalysed in synthesis in organic media: a practical procedure for organic chemists. *Tetrahedron*, 48(13), pp.2793–2802.
- Liu, H. et al., 2015. Radical Scavenging by Acetone: A New Perspective to Understand Laccase/ABTS Inactivation and to Recover Redox Mediator. *Molecules*, 20(11), pp.19907–19913.

Chapter 8

Lu, A.H., Salabas, E.L. & Schuth, F., 2007. Magnetic nanoparticles: Synthesis, protection, functionalization, and application. *Angewandte Chemie - International Edition*, 46(8), pp.1222–1244.

Paiva, A.L., Balcao, V.M. & Malcata, F.X., 2000. Kinetics and mechanisms of reactions catalyzed by immobilized lipases. *Enzyme and microbial technology*, 27(3), pp.187–204.

Romero, M.D. et al., 2005. Enzymatic synthesis of isoamyl acetate with immobilized *Candida antarctica* lipase in n-hexane. *Enzyme and microbial technology*, 37(1), pp.42–48.

Salah, R. Ben et al., 2007. Production of butyl acetate ester by lipase from novel strain of *Rhizopus oryzae*. *Journal of Bioscience and Bioengineering*, 103(4), pp.368–372.

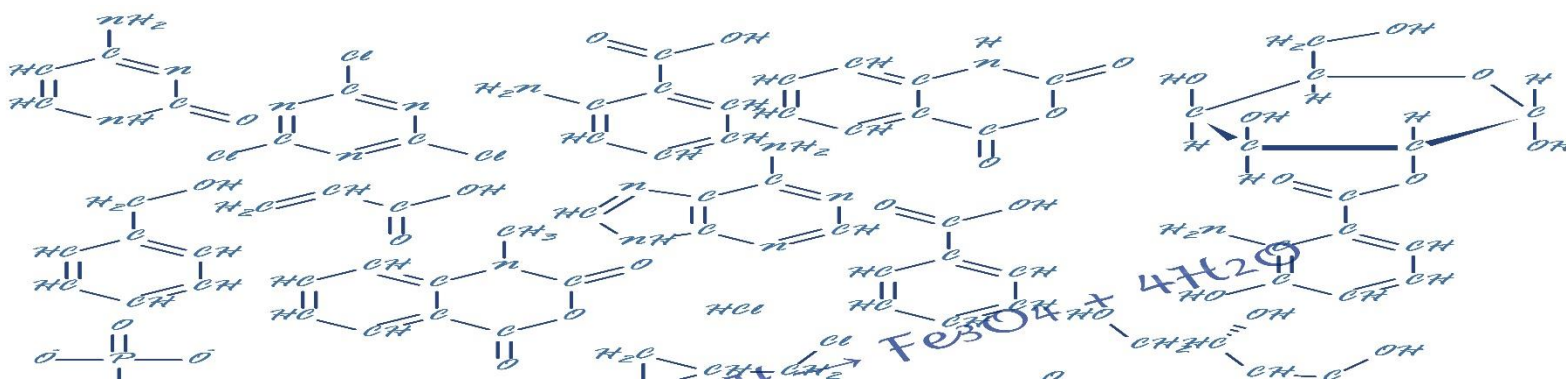
Singh, R.K. et al., 2013. From protein engineering to immobilization: Promising strategies for the upgrade of industrial enzymes. *International Journal of Molecular Sciences*, 14(1), pp.1232–1277.

Smith, P. et al., 1985. Measurement of protein using bicinchoninic acid. *Analytical biochemistry*, 150(1), pp.76–85.

Welsh, F.W. et al., 1989. Microbiological and enzymatic production of flavor and fragrance chemicals. *Critical Reviews in Biotechnology*, 9(2), pp.105–169.

Xie, W. & Ma, N., 2009. Immobilized lipase on Fe₃O₄ nanoparticles as biocatalyst for biodiesel production. *Energy & Fuels*, 23(3), pp.1347–1353.

Chapter 9



An improved method to measure
Lipase activity in aqueous media

Abstract

An improved method based on the *p*-nitrophenyl palmitate method is proposed to measure lipase hydrolytic activity in aqueous media. Using ethylene glycol as co-solvent for hydrophobic *p*-nitrophenyl substrates in aqueous buffer, lipase activity is measured following by the release of *p*-nitrophenol at 405 nm.

The method improves the solubility of substrate and product, also the stability of the substrate (in ethylene glycol the substrate was not hydrolyzed), avoids the use of organic solvents (ethanol, methanol or hexane) that could inhibit the enzyme and are difficult to handle, permits the comparison of all *p*-nitrophenol acyl esters substrates, allows the study of surfactants on the enzymes, and the influence of ions like Ca^{+2} can be studied without turbidity in the medium. The proposed method is fast, easy to handle, and linear in ample range of enzyme concentration 0-0.8 mg/mL.

Keywords: Lipase hydrolytic activity, *p*-nitrophenyl esters, ethylene glycol, *p*-nitrophenol, aqueous media.

1 Introduction

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are widely used in biotechnology for transesterification reactions, to prepare long and short chain esters, or as enantiomeric catalysts. Lipases are found in animals, plants (Mukherjee & Hills 1994), fungi (Iwai & Tsujisaka 1984) and bacteria (Jaeger et al. 1994), the most widely used in biotechnology being those obtained from microbial sources (Jaeger & Reetz 1998).

One of the particularities of lipases is that they are water soluble proteins that work on water insoluble or partially water insoluble substrates, which then become water insoluble products. It is this which makes lipase activity so difficult to measure.

Numerous methods are available to measure lipase hydrolytic activity (Brockerhoff & Jensen 1974), the most widely used being the titrimetric method with olive oil as substrate, in which the fatty acid released from the triglyceride by titration with potassium hydroxide is determined (Jensen 1983). However, the method has several disadvantages like the restricted pH range, the low sensitivity (up to 1 $\mu\text{mol}/\text{min}$) (Tiss et al. 2016), and the need to add emulsifiers such as arabic gum or surfactants (triton, brij, tween) to maintain the homogeneity of the reaction medium. The additives could interact with the lipase and act as activators or inhibitors of the enzyme.

The second most commonly used method is the colorimetric method using *p*-nitrophenyl palmitate as substrate, measuring the release of *p*-nitrophenol as a yellow chromophore (Hasan et al. 2009). The main advantage of this method is its simplicity, however it has as though does have some disadvantages such as the turbidity generated when the palmitate is released to the aqueous medium, or the need to add emulsifiers or organic solvents (ethanol or propanol) to maintain the homogeneity of the reaction medium. Organic solvents are difficult to measure and handle, and their low boiling points could cause their evaporation from the reaction medium.

Chapter 9

For its simplicity and easy handling we propose an improved method to measure lipase hydrolytic activity with *p*-nitrophenyl palmitate and its analogous *p*-nitrophenyl substrates of differing fatty acid chain lengths. Ethylene glycol, an organic compound widely using in industry, is a polar molecule (Carter et al. 1965), that is soluble in most organic solvents, with a high boiling point (195 °C) and low viscosity (1.1 g/L). These properties make it a good candidate to act as co-solvent between the water, the hydrophobic *p*-nitrophenyl esters and the released fatty acids.

Using ethylene glycol as co-solvent, the substrates are solubilized and can be mixed with water. As the fatty acid released is also soluble, it does not interfere with the measurement. The release *p*-nitrophenol is followed spectrophotometrically at 405 nm.

2 Materials and Methods

2.1 Materials

Lipase from *Mucor javanicus* and *Pseudomonas fluorescens* were obtained from Sigma Aldrich. The used substrates *p*-nitrophenyl butyrate, caprylate, caprate, laurate, mirystate and palmitate were purchased from Sigma Aldrich, as was the ethylene glycol used in the assays.

2.2 Methods

To test the improved method for lipase hydrolytic activity in aqueous media, the most influential parameters in the lipase activity had been studied and optimized for two selected lipases *Mucor javanicus* and *Pseudomonas fluorescens*, enzyme mass, optimal concentration of ethylene glycol, pH, temperature and kinetic parameters. Also for *Pseudomonas fluorescens* the effect of additives, like Ca^{+2} was also studied.

2.2.1 *p*-nitrophenol calibration curve

Calibration curves of *p*-nitrophenol were obtained in 50 mM tris/acetate buffer pH 6.5, one with 5 % of ethylene glycol and one without it, using increasing quantities of 10 μM *p*-nitrophenol stock solution prepared in 50 mM tris/acetate buffer pH 6.5. The absorbance of the samples was measured at 405 nm and the results fitted to a straight line equation.

2.2.2 Preparation of Substrate Stock solutions

The *p*-nitrophenyl substrates (butyrate, caprylate, caprate, laurate, mirystate and palmitate) were dissolved in ethylene glycol in a water bath sonicator (Branson Ultrasonics Danbury, USA) at 500 mM each, to prepare the substrate stock solutions. These solutions remain stable for several weeks at 4°C.

2.2.3 Lipase activity assay optimization

To test the improved method for measure lipase hydrolytic activity in aqueous media and to validate the use of ethylene glycol as hydrophilic solvent for *p*-nitrophenyl substrates, the most influential parameters in the lipase activity enzyme mass, optimal concentration of ethylene glycol, pH, temperature and kinetic parameters were studied and optimized for two lipases, *Mucor javanicus* and *Pseudomona fluorescens*. In the case of *Pseudomona fluorescens* the effect of Ca⁺² trybutirin, SDS and Triton X-100 were also studied. Kinetic characterization for both lipases with the six substrates were made in the best conditions obtained in the optimization.

2.2.4 Lipase general activity assay

Lipase activity was measured with *p*-nitrophenyl ester substrates, following our improved method. Stock solutions of these substrates (500 mM) were adjusted to 25 mM with 50 mM tris/acetate pH 6.5, the final concentration of ethylene glycol being 5 %. In a 1 mL spectrophotometer cuvette, 880 µL of tris/acetate buffer (50 mM pH 6.5, 5 % ethylene glycol) and 100 µL of the prepared substrate were added, and the reaction was started by adding 20 µL of lipase (1 mg/mL). The formation of the yellow compound *p*-nitrophenol was measured at 405 nm in a Shimadzu UV 2401 PC spectrophotometer (Shimadzu Corporation Japan) equipped with a temperature controller Shimadzu TCC Controler 24017 set at 30 °C.

To enable the comparison between enzymes all the assays except the kinetics assays were made using *p*-nitrophenyl caprate as substrate.

2.2.5 Effect of enzyme mass

To study the effect of enzyme in the reaction, different masses of *P. fluorescens* lipase and *M. javanicus* were used, keeping the other parameters constant: pH (tris/acetate 50 mM pH 6.5) temperature (30 °C), ethylene glycol concentration (5 %) and substrate concentration (2.5 mM).

2.2.6 Optimization of ethylene glycol

Several final concentrations of ethylene glycol between (0- 10 %) were tested for the two lipases, using *p*-nitrophenyl caprolate (2.5 mM). Enzyme mass (20 µg), pH (50 mM tris/acetate pH 6.5) and temperature (30 °C) were maintained constant during the assays.

2.2.7 Temperature and pH optimization

A range of temperatures (25-70 °C) was tested to determine the optimal working temperature for the enzymes and also to test the stability of the reaction medium. Which contained 50 mM tris/acetate buffer pH 6.5, 2.5 mM of substrate, 5 % of ethylene glycol and 20 µg of enzyme.

To ascertain the optimal pH, 50 mM solutions of tris/acetate (pH 6.5-9.0) or acetic acid/acetate (pH 5.0-4.0) were tested, while keeping the rest of the parameters constant: temperature at its respective optimal value, 5 % ethylene glycol, 2.5 mM of substrate and 20 µg of lipase.

2.2.8 Kinetic studies

Kinetic studies were made for the two lipases with the six 4-nitrophenyl substrates. In these assays the pH (50 mM tris/citrate pH 6.5 for *M. javanicus* and 7.5 for *P. fluorescens*), temperature (25 °C for *P. fluorescens* and 60°C for *M. javanicus*) and ethylene glycol concentration (5 %) were maintained constant as was the enzyme mass (20 µg). In the kinetic assays of *P. fluorescens* 15 mM of CaCl₂ was also added to the

reaction medium. The results were fitted to the Michaelis-Menten equation, to obtain the kinetic parameters (K_M and V_{MAX}).

2.2.9 Effect of additives

Is know the positive effects that surfactants as triton X-100 or substrates like tributyrin causes to lipases in aqueous media. Knowing that, several compounds were tested in different concentrations to assets their effect in the lipase hydrolytic activity, and to test if the method interfere in the assay.

The compounds tested were Ca^{+2} (0-100 mM) Triton X-100 solution (10 %), Sodium dodecyl sulfate (SDS) solution (10 %), polyethylene glycol 600 (PEG 600), tributyrin and benzyl alcohol. The final volume in cuvette were 0, 0.25, 1, 5 %, pH (50 mM tris/citrate pH 7.5), temperature (25 °C), mass of enzyme (20 μ g), *p*-nitrophenyl myristate (2.5 mM) and ethylene glycol were maintained constant.

3 Results and Discussion

3.1 *p*-nitrophenol calibration curve

The presence of 5 % ethylene glycol did not change the calibration curve of *p*-nitrophenol as shows Fig 1. The molar extinction coefficient (ϵ) obtained at pH 6.5 was $11267 \text{ M}^{-1} \text{ cm}^{-1}$ ($R^2 0.9994$), and without ethylene glycol it was $11253 \text{ M}^{-1} \text{ cm}^{-1}$ ($R^2 0.9995$), a value close enough to assume that the ethylene glycol did not interact with the chromophore.

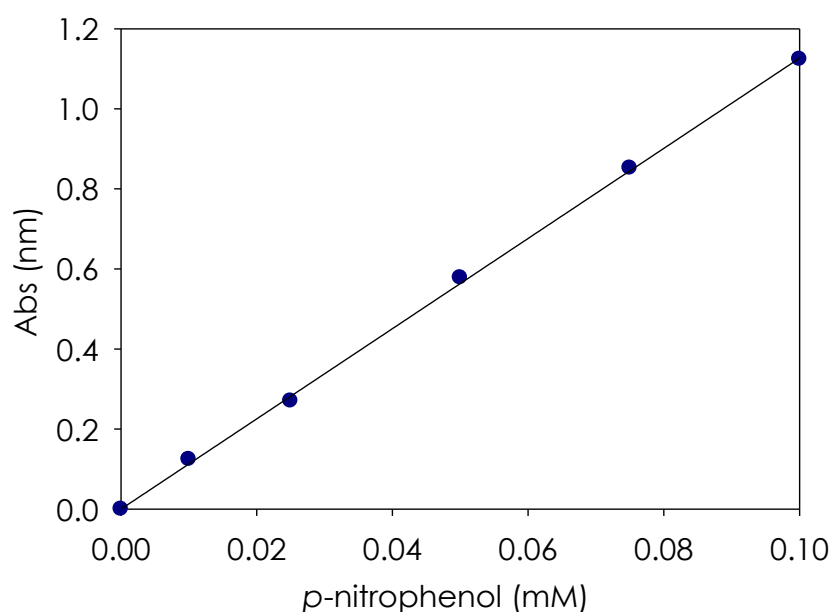


Fig 1: Calibration curve of *p*-nitrophenol at pH 6.5 (50 mM tris/acetate) and 5% ethylene glycol.

3.2 Enzyme mass

The effect of the enzyme mass showed that *M. javanicus* the response was linear up to 0.8 mg of enzyme, while for *P. fluorescens* the method was linear only to 0.2 mg.

3.3 Ethylene glycol effect

As depicted in Fig 2 the enzymatic activity increased when ethylene glycol was added, as a result of the interaction between the compound and the enzyme, probably due to an improved structural conformation of the enzyme. The activation of lipases due to changes between open-lid conformational form (activated) and closed-lid form, is well known (Fernandez-Lafuente et al. 1998), and could be attained by treating the enzyme with polar solvents (Chamorro et al. 1998). Ethylene glycol is also a polar solvent (Carter et al. 1965) so the activation results that shows the Fig 2 were expected. There was a loss of activity when the quantity of ethylene glycol surpassed 10 % that was more pronounced in *P. fluorescens* than in *M. javanicus*, while the activity increase was higher, indicating the need to optimize this parameter for individual lipases. For the rest of the work the percentage of cosolvent was 2 % for *M. javanicus* and 5 % for *P. fluorescens*. However for the rest of the work ethylene glycol was fixed in 5 %.

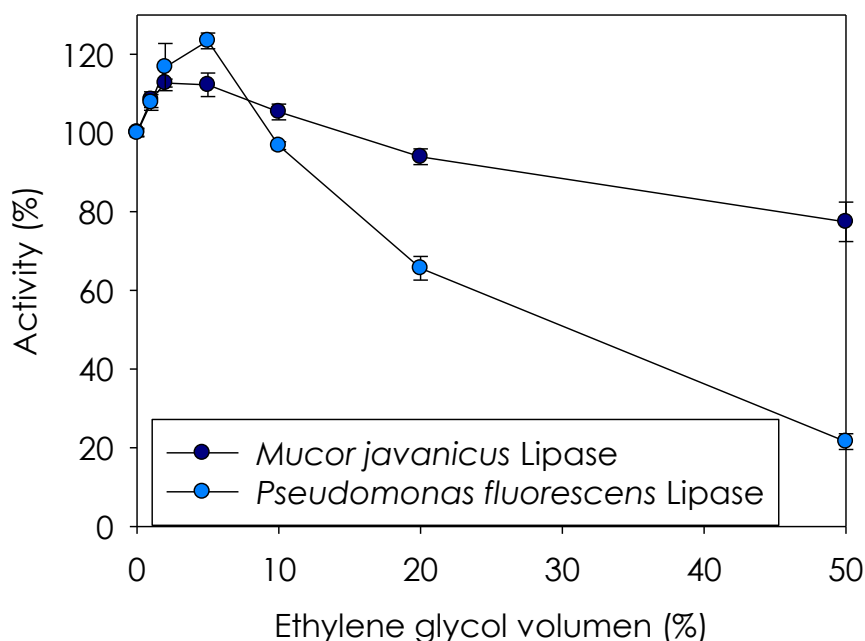


Fig 2: Effect of ethylene glycol in lipase hydrolytic activity, 20 μ g of lipase, at pH 6.5 (tris/acetate 50 mM), 2.5 mM of substrate and 30 $^{\circ}$ C.

3.4 Temperature and pH profiles

The Fig 3 shown the temperature effect on the enzymes, the temperature profile of *P. fluorescens* was almost constant, with a slight decrease of activity above 50 °C, whilst the activity of *M. javanicus* Lipase increase with the temperature, with a clear maximum at 60 °C. The pH effect on the lipases activity (Fig 4), pointed to a pH optimum 7.5 for *P. fluorescens* and 6.5 for *M. javanicus*. The data obtained for *P. fluorescens* was in total agreement with the literature, which reports a stable temperature range between 5 and 50 °C with a maximum at 20-25 °C and pH between 7.0 and 8.0 (Luo et al. 2006), this was similar to (Salis et al. 2005), underlining the few interactions that the co-solvent has with the enzymes and showing our method can be compared with other methods for measuring activity. Compared with other polar solvents normally used, such as ethanol or propanol, ethylene glycol has a higher boiling point (195-198 °C) than both, avoiding solvent evaporation when working at high temperature.

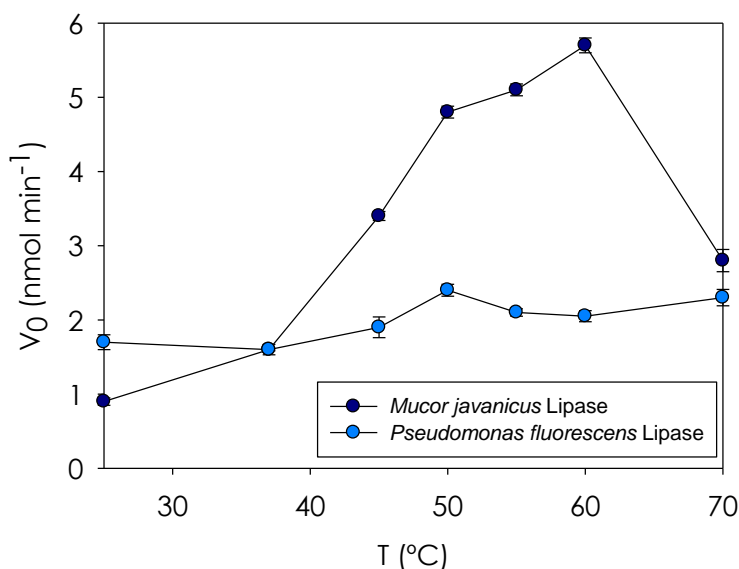


Fig 3: Temperature profiles of lipases (20 µg) using 2.5 mM of substrate, 5 % ethylene glycol and pH 6.5 (tris/acetate 50 mM).

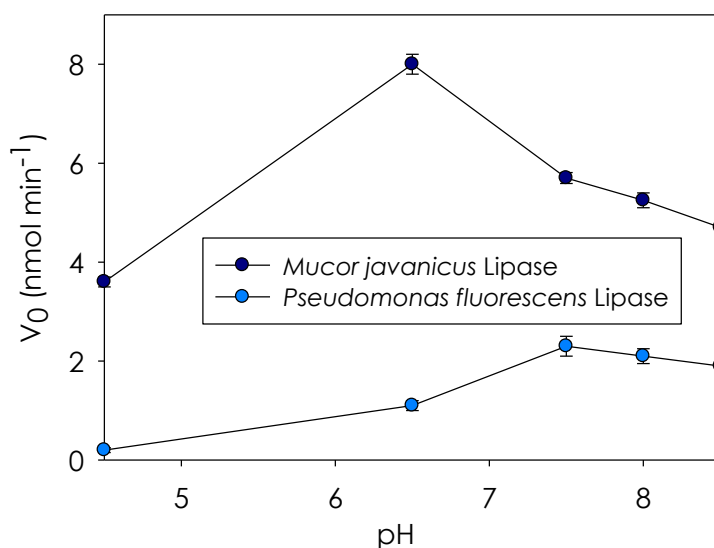


Fig 4: Effect of the pH for the lipases, using the optimal temperature from above lipases (20 μg) using 2.5 mM of substrate and 5 % ethylene glycol.

3.5 Kinetic parameters

The kinetic parameters for all *p*-nitrophenyl esters are summarized in Table 1. In the case of *M. javanicus* the substrate with most affinity was *p*-nitrophenyl palmitate since it had a lower K_M . In the case of *P. fluorescens* the substrate with most affinity *p*-nitrophenyl myristate.

Table 1: Kinetic parameters for the *P. fluorescens* and *M. javanicus* for the six *p*-nitrophenyl esters.

Substrate	C	<i>Mucor javanicus</i>			<i>Pseudomonas fluorescens</i>		
		V_{MAX} (nmol min ⁻¹)	K_M (mM)	AE (nmol min ⁻¹ mg ⁻¹)	V_{MAX} (nmol min ⁻¹)	K_M (mM)	AE (nmol min ⁻¹ mg ⁻¹)
Butyrate	4	67.8	4.3	3.39	10.7	5.73	0.54
Caprylate	8	103.52	5.0	5.18	19.6	5.3	0.98
Caprate	10	30.8	6.88	1.54	13.65	10.6	0.68

Laurate	12	53.98	11.49	2.7	12.69	9.7	0.63
Mirystate	14	15.04	1.35	0.752	4.1	0.57	0.21
Palmitate	16	6.4	0.95	0.32	4.0	0.88	0.20

3.6 Effect of additives

P. fluorescens lipase has been reported to be calcium dependent, since Ca^{+2} induced secondary structural changes that improved the activity (Kim et al. 2005). However the Ca^{+2} ions present in the medium react with the released fatty acids, forming insoluble calcium esters that tend to cloud the medium (Tigerstrom & Stelmaschuk 1989) and hinder the colorimetric measurements. As shown Fig 5, 15 mM was the optimum calcium concentration in the case of *P. fluorescens*, although the method was tested up to 100 mM of Ca^{+2} using *p*-nitrophenyl palmitate as substrate, without showing any turbidity and enabling the release of *p*-nitrophenol to be measured without problems.

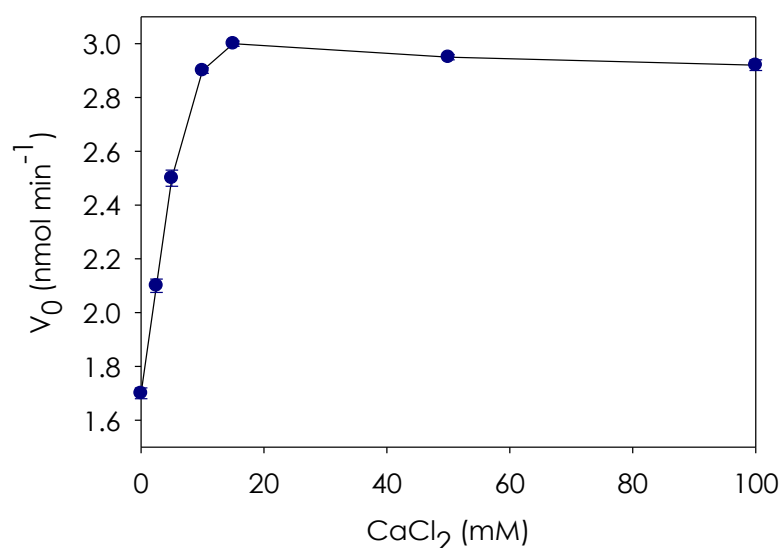


Fig 5: Effect on calcium on the *P. fluorescens* lipase activity, at 25 °C, pH 7.5, 5 % of ethylene glycol, and 2.5 mM of *p*-nitrophenyl palmitate

The effect of additives towards the lipase activity was evaluated in Fig 6, as could be seen 0.25 % of triton X-100 improved the lipase activity 2.5 fold whilst 10 % of SDS reduced the activity to 10 %. Common lipase substrates as benzyl alcohol or tributyrin improved slightly the activity, probably the polarity of benzyl alcohol or the hydrophobicity of tributyrin enhanced the active structure of the enzyme (Fernandez-Lafuente et al. 1998). Also, the polyethylene glycol ₆₀₀ improved the activity, indicating that any (poly) ethylene glycol can be used in the method as well, although the medium will be more viscous than with ethylene glycol.

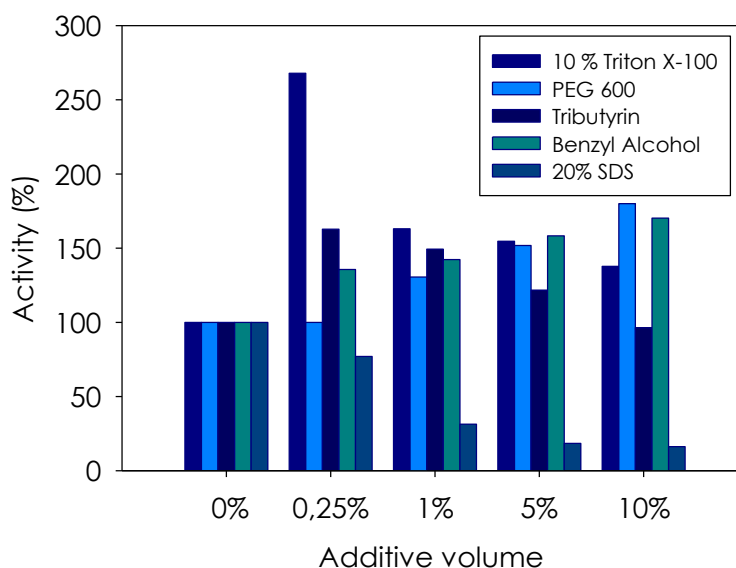


Fig 6: Effect of additives the *P. fluorescens* lipase activity, at 25 °C, pH 7.5, 5 % of ethylene glycol, and 2.5 mM of *p*-nitrophenyl palmitate.

4 Conclusions

Using an economically affordable co-solvent, and hydrophobic *p*-nitrophenyl esters as substrates lipase hydrolytic activity could be measured in aqueous buffer. We have demonstrated that the co-solvent does not interfere in the measurements, and slightly improves the lipase activity but does not interact with the released chromophore, Ca^{+2} ions or buffers. The boiling point of the compound enables activity to be measured at highest temperatures, which is not possible with other organic solvents as they evaporate. The use of *p*-nitrophenyl esters permits a fast screening for the best enzyme substrate and also shows whether the enzyme is a pure lipase, which favors long chain esters such as palmitate, or an esterase which would prefer short chain esters like butyrate.

5 References

- Brockerhoff, H. & Jensen, R.G., Lipolytic Enzymes (1974). *New York*, pp.10–24.
- Carter, D.L., Heilman, M.D. & Gonzalez, C.L., 1965. Ethylene glycol monoethyl ether for determining surface area of silicate minerals. *Soil Science*, 100(5), pp.356–360.
- Chamorro, S. et al., 1998. Treatment of *Candida rugosa* lipase with short-chain polar organic solvents enhances its hydrolytic and synthetic activities. *Biotechnology Letters*, 20(5), pp.499–505.
- Fernandez-Lafuente, R. et al., 1998. Immobilization of lipases by selective adsorption on hydrophobic supports. *Chemistry and Physics of Lipids*, 93(1), pp.185–197.
- Hasan, F., Shah, A.A. & Hameed, A., 2009. Methods for detection and characterization of lipases: a comprehensive review. *Biotechnology advances*, 27(6), pp.782–798.
- Iwai, M. & Tsujisaka, Y., 1984. Fungal lipase. In *Lipases*, Academic Press, New York pp.443–469.
- Jaeger, K.-E. et al., 1994. Bacterial lipases. *FEMS microbiology reviews*, 15(1), pp.29–63.
- Jaeger, K.-E. & Reetz, M.T., 1998. Microbial lipases form versatile tools for biotechnology. *Trends in biotechnology*, 16(9), pp.396–403.
- Jensen, R.G., 1983. Detection and determination of lipase (acylglycerol hydrolase) activity from various sources. *Lipids*, 18(9), pp.650–657.
- Kim, K.R. et al., 2005. Purification, refolding, and characterization of recombinant *Pseudomonas fluorescens* lipase. *Protein expression and purification*, 39(1), pp.124–129.
- Luo, Y. et al., 2006. A novel psychrophilic lipase from *Pseudomonas fluorescens* with unique property in chiral resolution and biodiesel production via transesterification. *Applied Microbiology and Biotechnology*, 73(2), pp.349–355.

Chapter 9

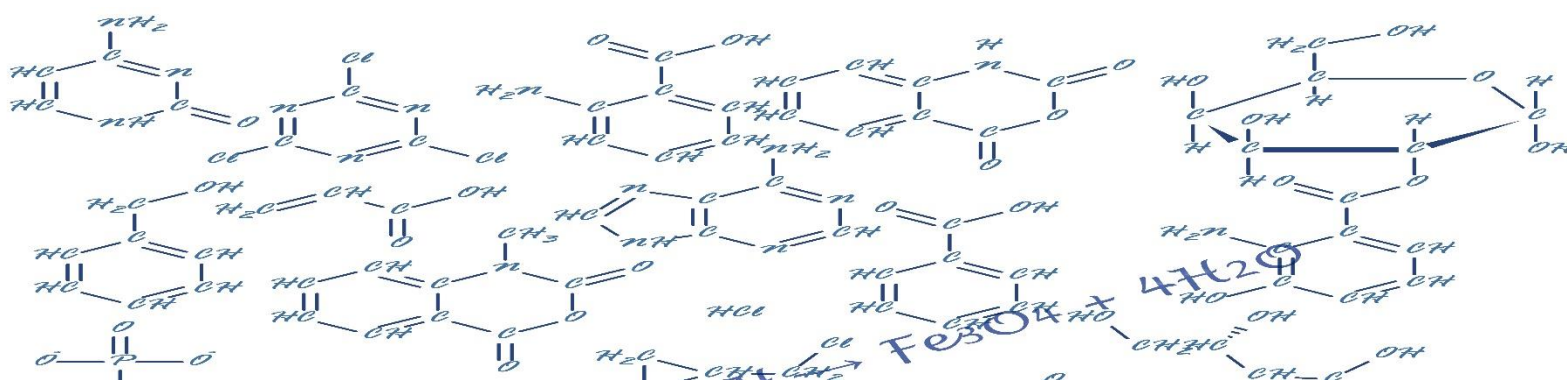
Mukherjee, K.D. & Hills, M.J., 1994. Lipases from plants. *Lipases: their structure, biochemistry and application*, pp.49–75.

Salis, A. et al., 2005. Physical and chemical adsorption of *Mucor javanicus* lipase on SBA-15 mesoporous silica. Synthesis, structural characterization, and activity performance. *Langmuir*, 21(12), pp.5511–5516.

Tigerstrom, R.G. von & Stelmaschuk, S., 1989. The use of Tween 20 in a sensitive turbidimetric assay of lipolytic enzymes. *Canadian journal of microbiology*, 35(4), pp.511–514.

Tiss, A., Beisson, F. & Rivière, C., 2016. Methods for Lipase Detection and Assay : A Critical Review. *European Journal of Lipid Science and Technology*, 120, pp. 133-153.

Chapter 10



Absorbance and Fluorescence studies
of Anthranilic, 3-Hydroxyanthranilic
and Cinnabarinic Acids.

Abstract

Anthranilic acid, 3-hydroxyanthranilic acid and cinnabarinic acid are three related compounds that appear in the kynurenine pathway, they are important metabolites in the inflammation and autoimmune processes, as well as markers of wide range of clinical diseases such as chronic brain injury, Huntington's disease, stroke, depression, or osteoporosis.

Being important metabolites and biomarkers for diseases, their spectroscopic and fluorescence characteristics had been described, as well as their water affinity. The results showed that absorbance and fluorescence of these compounds is strongly related to pH, showing one or more pK_as in the studied pH range. The three compounds showed fluorescence, however cinnabarinic acid fluorescence was weak and the compound tends to decompose with the incident light. The water affinity studies showed that anthranilic acid had the lowest affinity for water, having an affinity constant of 49.9 M.

Keywords: Absorbance, fluorescence, anthranilic acid, 3-hydroxyanthranilic acid, cinnabarinic acid.

1 Introduction

Anthranilic, 3-hydroxyanthranilic and cinnabarinic acids, are three related compounds with similar structures as can be seen in Fig 1. 3-hydroxyanthranilic acid is the hydroxylated anthranilic acid in position three and cinnabarinic acid is the oxidation-dimerization product of 3-hydroxyanthranilic acid.

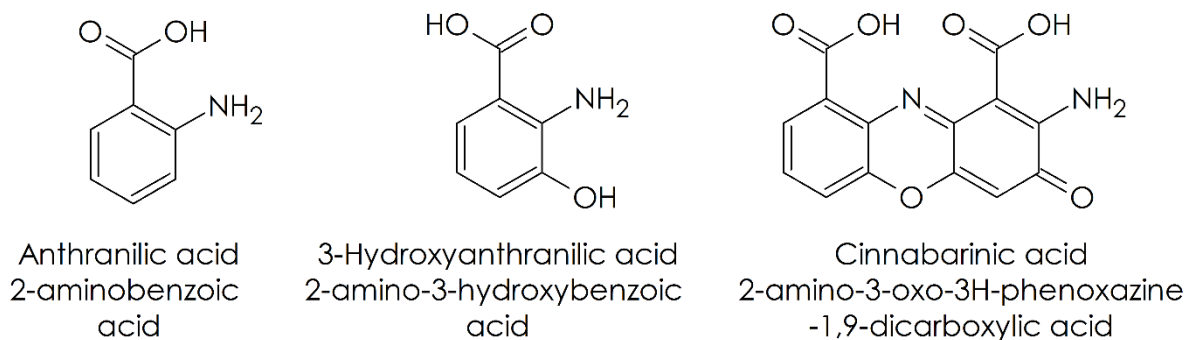


Fig 1: Anthranilic, 3-hydroxyanthranilic and cinnabarinic acid chemical structures.

Anthranilic acid has been used as fluorescent label for glycans (Bigge et al. 1995) due its small size, long stokes shift in water and high quantum yield ($\Phi = 0.6$). Anthranilic acid appears in nature in the nematode *Caenorhabditis elegans* intestinal lysosome-related organelles, it seems to be related to the aging and the necrotic wave that promotes the death of the organism (Coburn et al. 2013; Gems & Coburn 2013) appearing as a blue fluorescence burst before the death (Fig 2). Cinnabarinic acid and 3-hydroxyanthranilic acid are secondary metabolites of the kynurenine pathway (Fazio et al. 2012) related to inflammation and autoimmune processes. Clinical data from patients with wide range of clinical diseases—chronic brain injury, Huntington’s disease, stroke, depression, coronary heart disease, intrathoracic disease including neoplasia and osteoporosis the normal ratio between 3-hydroxyanthranilic acid and anthranilic acid is changed, with lower levels of 3-hydroxyanthranilic acid and higher levels of anthranilic acid than normal (Darlington et al. 2010).

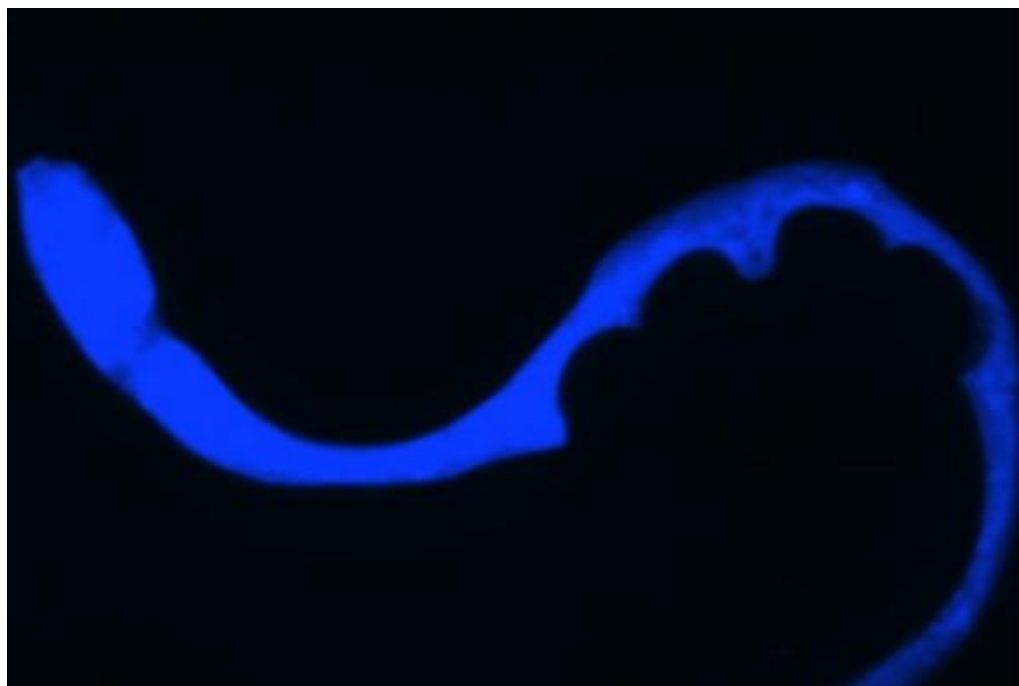


Fig 2: *C. elegans* blue “death wave” caused by anthranilic acid.

As mentioned earlier anthranilic acid fluorescence and its use as fluorescent label has been reported, 3-hydroxyanthranilic acid fluorescence has been reported (McMillan 1960) but not described, and cinnabarinic acid has not been studied. Being important metabolites present biological pathways, it is important to have a detailed study of its spectroscopy properties (fluorescence and absorbance), for them to be detected and measured or to be used as potential fluorophores for detection and labeling of biomolecules.

The spectroscopy and fluorescent properties of the three compounds have been described: absorbance and fluorescence spectra, behavior in water or organic solvents, the effect of pH in absorbance and fluorescence and the effect of water in the spectroscopy was also studied using a binary system which allows to determine the affinity of the molecules towards water.

2 Materials and Methods

2.1 Materials

The used reagents anthranilic acid (reagent grade > 98%), and cinnabarinic acid were obtained from Sigma Aldrich, 3-hydroxyanthranilic acid was purchased from TCI (TCI Belgium). The rest of the reagents used in the work were also obtained from Sigma Aldrich of reagent grade and used without further purification.

2.2 Methods

Stock solutions of the reagents anthranilic acid, 3-hydroxyanthranilic acid and cinnabarinic acid were prepared at 1 mM in methanol this solutions are stable for months kept at -20 °C.

2.2.1 Relationship between the absorbance of anthranilic, hydroxyanthranilic and cinnabarinic acids and pH

Phosphoric acid (40 mM), boric acid (40 mM) acetic acid (40 mM) and NaOH (200 mM) were used to prepare Britton-Robinson (BR) aqueous universal buffer solutions in a pH range between 2 and 12 (Britton 1932). The measures were made in a 1 mL spectrophotometric cuvette containing 995 μ L of buffered solution and 5 μ L of the compounds stock solutions, the absorbance spectrum was recorded at wavelengths between 220 and 550 nm.

The maximum peak absorbance wavelength obtained in the spectra was plotted versus the pH and fitted to a four parameters sigmoidal curve with Sigma Plot to obtain the compound pKs.

2.2.2 Water affinity of anthranilic, hydroxyanthranilic and cinnabarinic acids

The affinity for water was studied preparing several solutions with different molarities of water in DMSO, 995 μL of the water/DMSO solution and 5 μL of the reagents stock solutions were mixed and the absorbance recorded at wavelengths between 220 and 550 nm.

The absorbance of the spectra at one specific wavelength was plotted versus the molarity of water (Abou-Zied et al. 2013). The curve was adjusted to Eq.1 a four parameter Hill equation (Hill 2013), being x the water concentration in the binary mix, y the recorded absorbance, K the affinity constant, and b the Hill coefficient.

$$y = \frac{y_0 \cdot a \cdot K^{-b} \cdot x^b}{1 + K^{-b} \cdot x^b} \quad (\text{Eq.1})$$

2.2.3 Fluorescence spectra of anthranilic, 3-hydroxyanthranilic and cinnabarinic acids

Excitation and emission spectra for the compounds were recorded in a Shimadzu spectrofluorimeter RF 6000 (Shimadzu Japan). The excitation spectra were recorded fixing the emission in 410 nm, and emission spectra were recorded using the maximum peak wavelengths of the excitation spectra. The assays consist in 10 μM of the compound in water using a 3 mL fluorescence quartz cuvette.

2.2.4 Fluorescence of anthranilic, 3-hydroxyanthranilic and cinnabarinic acids with different pH

Phosphoric acid (40 mM), boric acid (40 mM) acetic acid (40 mM) and NaOH (200 mM) were used to prepare used buffered solutions in a pH range between 2 and 12. In a 0.3 mL microplate 298 μ L of buffered solution and 2 μ L of stock solution was mixed and the fluorescence recorded in a Synergy microplate reader (BioTek) with the filter 360/460 ex/em for anthranilic and 3-hydroxyanthranilic and cinnabarinic acid with the sensibility fixed in 75.

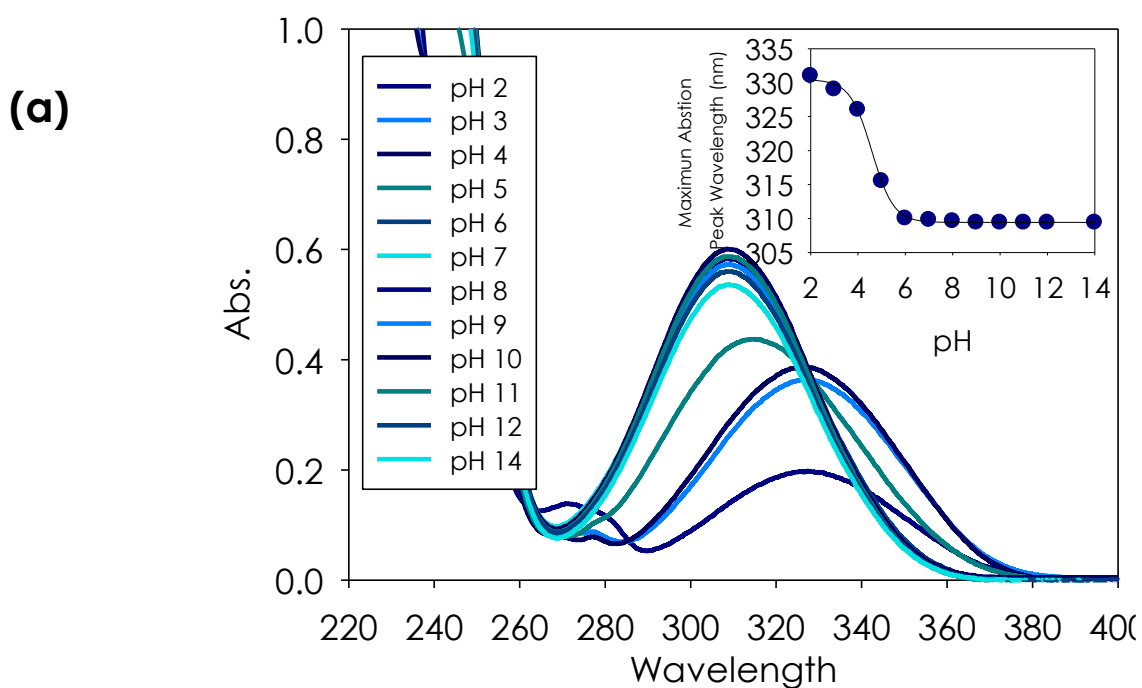
2.2.5 Fluorescence of anthranilic, 3-hydroxyanthranilic and cinnabarinic acids in different solvents

In order to study the fluorescence behavior of the compounds, two microliters of stock solution was mixed with 298 μ L of reagents stock solutions and the fluorescence was measured in a plate reader using the 360/460 filter for anthranilic, 3-hydroxyanthranilic and cinnabarinic acids.

3 Results and Discussion

3.1 Relationship between the absorbance of anthranilic, 3-hydroxyanthranilic and cinnabarinic acids and pH

There was a strong relationship between the pH and the absorbance of the compounds, the shape and the maximum absorbance wavelength changed with the pH. This was indication of a pKa titration in the pH range as can be verify in the plots (Fig 3 (a), (b) and (c)), anthranilic acid had one pKa (4.58) in the pH range whilst 3-hydroxyanthranilic acid (5.2 and 9.4) and cinnabarinic acid (4.5 and 8.3) had two pKas in the studied pH range.



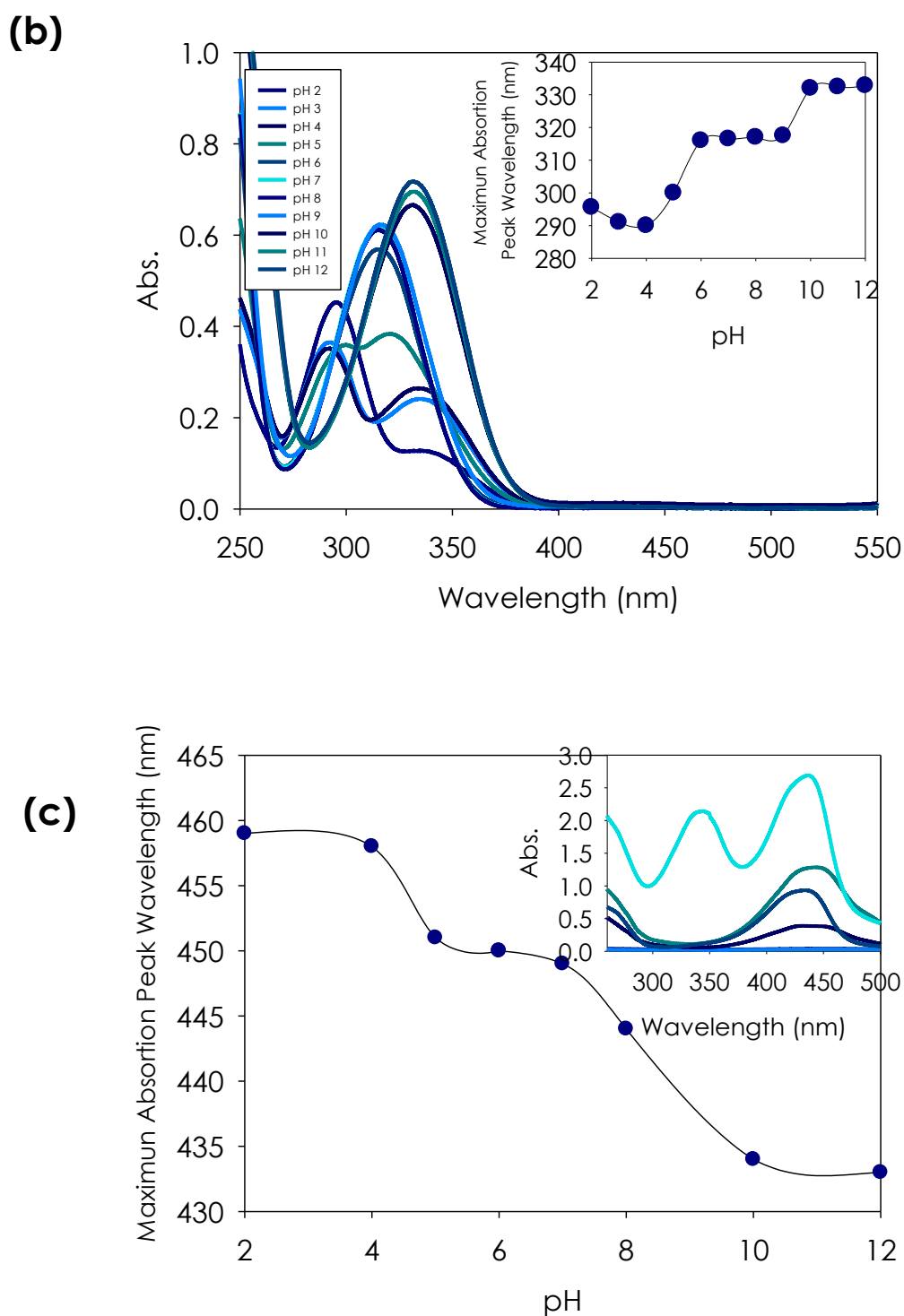
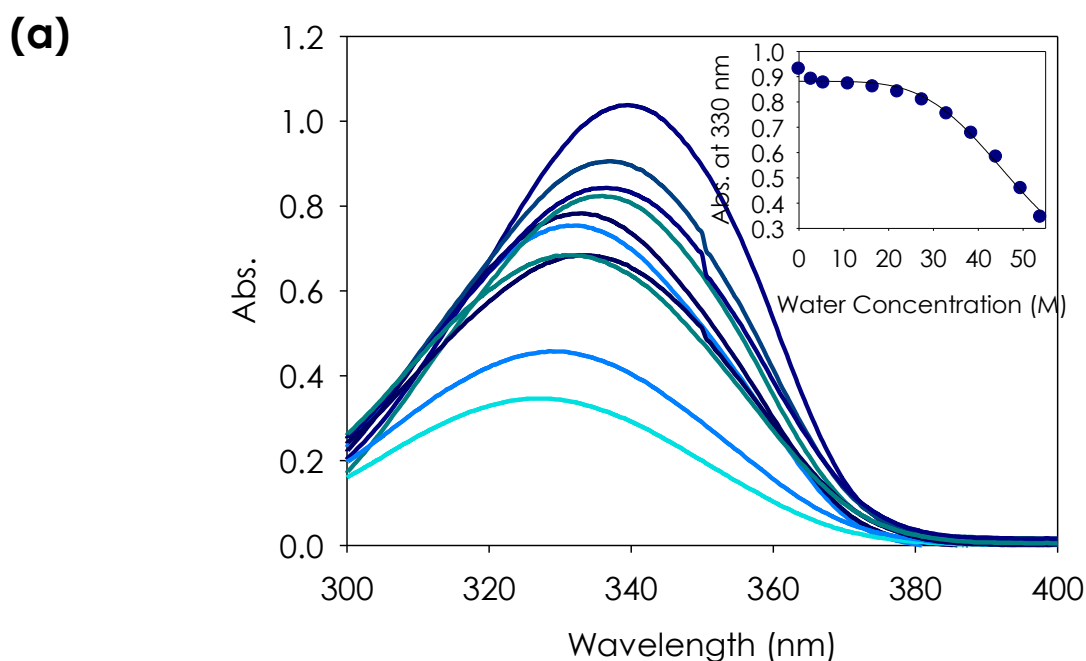


Fig 3: (a) Anthranilic pH spectra, in the inset pKa plot, (b) 3-hydroxyanthranilic pH spectra, in the inset pKa plot, and (c) cinnabaric acid pKa plot, in the inset the pH spectra is show.

3.2 Water affinity of anthranilic, hydroxyanthranilic and cinnabaric acids

Water affinity of the compounds was calculated using spectra of the compounds in binary mixtures of water/DMSO. Dimethyl sulfoxide was chosen because it is miscible in water, has no hydrogen-bond donor ability but has hydrogen-bond acceptor ability (Abou-Zied et al. 2013). The fit of the plots (Fig 4 (a), (b) and (c)) showed that cinnabaric acid had more affinity for water than the others compounds, with an affinity constant of 16.14 M and a Hill coefficient of 5.7. The second compound with most affinity for water was 3-hydroxyanthranilic acid with a constant of 46.19 M and a Hill coefficient of 5.3, anthranilic acid was the less affine, having the highest constant 49.9 M and the smaller Hill coefficient 4.4.



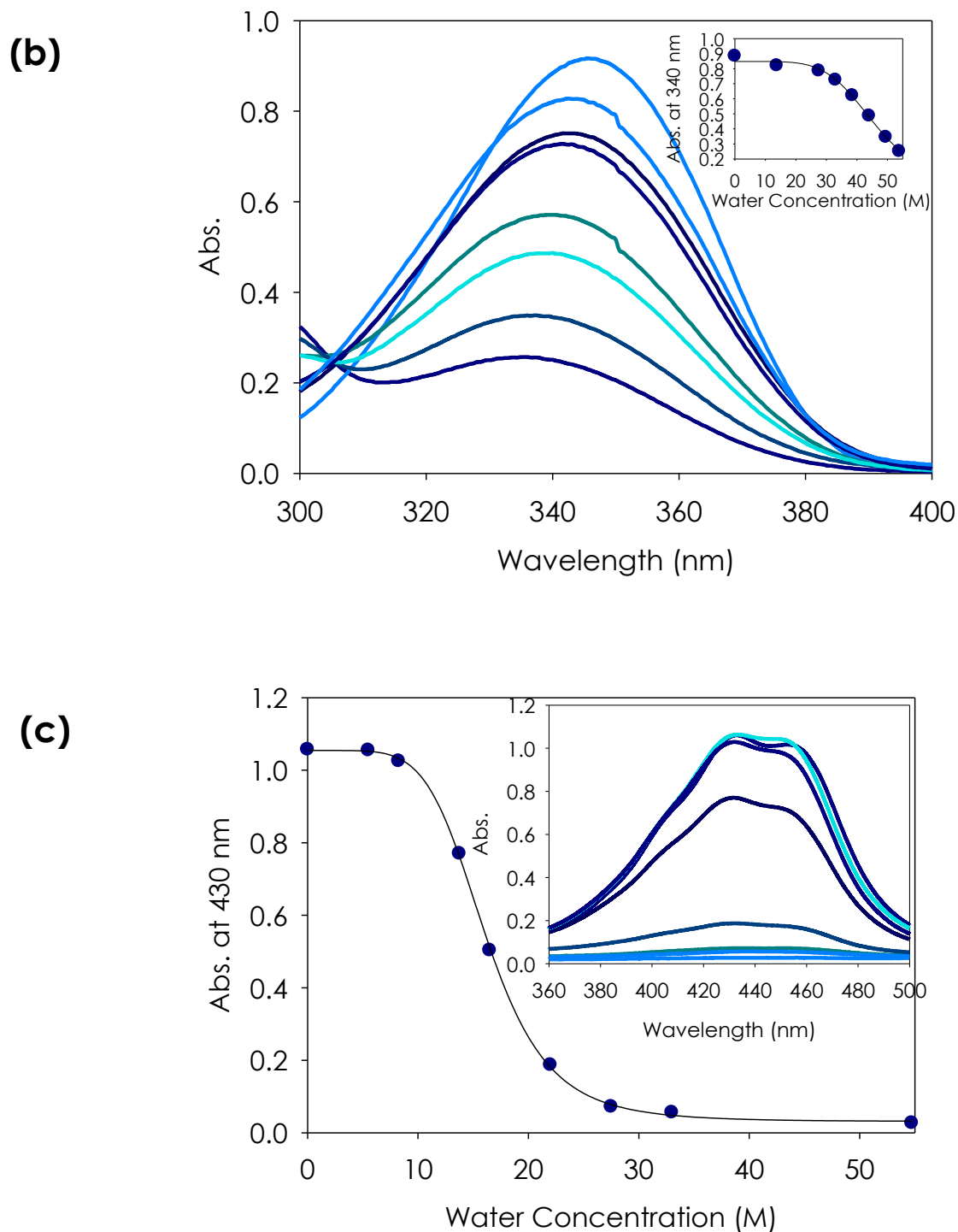


Fig 4: Water affinity spectra and water affinity plots for: **(a)** Anthranilic, **(b)** 3-hydroxyanthranilic, and **(c)** cinnabarinic acid.

As the can be seen in the spectra, the absorbance of the compounds was higher in DMSO than water, most probably because the molecules were more rigid in organic solvent than in water.

3.3 Fluorescence spectra of anthranilic, 3-hydroxyanthranilic and cinnabarinic acids

The fluorescence excitation and emission spectra for the three acids were recorded, as can be seen the excitation and emission spectra for the anthranilic (Fig 5) and 3-hydroxyanthranilic acid (Fig 6) are very similar, two excitation peaks situated at 220 and 315 nm and an emission peak at 410 nm. Nevertheless the excitation wavelength that gives the highest emission spectra differs between the compounds, the 315 nm peak gives the maximum emission peak for the anthranilic acid and the 220 nm peak gives much more emission than the 315 nm peak for 3-hydroxyanthranilic acid. As the figures show the compounds did not adhere to the mirror image rule, the excitation spectra was not the mirror image of the emission spectra. The ultraviolet absorption peak (220 nanometers) is due to an excitation transition to the second excited state (from S_0 to S_2) that quickly relaxes to the lowest excited state (S_1). As a consequence, fluorescence emission occurs exclusively from the lowest excited singlet state (S_1), known as the Kasha rule, resulting in a spectrum that mirrors the ground to first excited state transition (315 nanometer peak) in the compounds and not the entire absorption spectrum.

The energy associated with fluorescence emission transitions was typically less than that of absorption, the resulting emitted photons have less energy and are shifted to longer wavelengths. This phenomenon is generally known as Stokes Shift and occurs for virtually all fluorophores commonly employed in solution investigations. The primary origin of the Stokes shift is the rapid decay of excited electrons to the lowest vibrational energy level of the S_1 excited state. In addition, fluorescence emission is usually accompanied by transitions to higher vibrational energy levels of

the ground state, resulting in further loss of excitation energy to thermal equilibration of the excess vibrational energy.

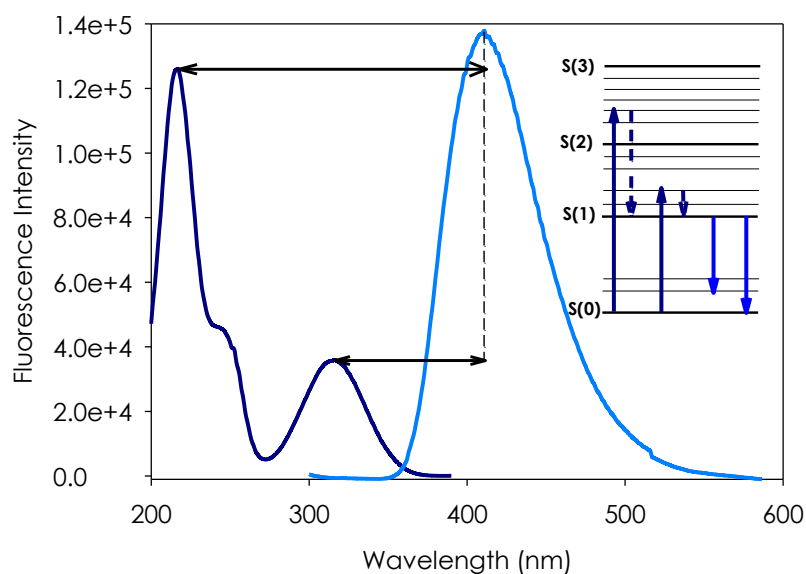


Fig 5: Anthranilic acid emission and excitation spectra in water, the inset shows the proposed Jablonski diagram.

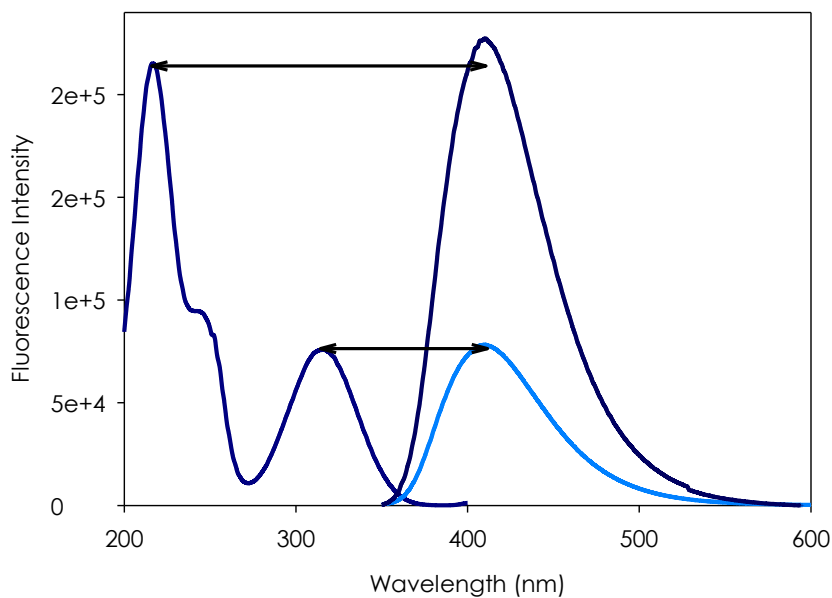


Fig 6: 3-hydroxyanthranilic acid emission and excitation fluorescence spectra in water.

In practice, the Stokes shift is measured as the difference between the maximum wavelengths in the excitation and emission spectra of a particular fluorochrome or fluorophore. The size of the shift varies with molecular structure, but can range from just a few nanometers to over several hundred nanometers. For example, the Stokes shift for fluorescein is approximately 20 nanometers, while for the porphyrins is over 200 nanometers. As depicted in Fig 5 and Fig 6 the shift for anthranilic and 3-hydroxyanthranilic acid was around 100 or 190 nanometers. The existence of Stokes shift is critical to the extremely high sensitivity of fluorescence imaging measurements.

Cinnabarinic acid has the less fluorescence of the three compounds tested, as shown in Fig 7, the spectra had two excitation peaks at 250 and 330 nm and an emission peak at 404 nm, having a Stokes shift of 74 nm.

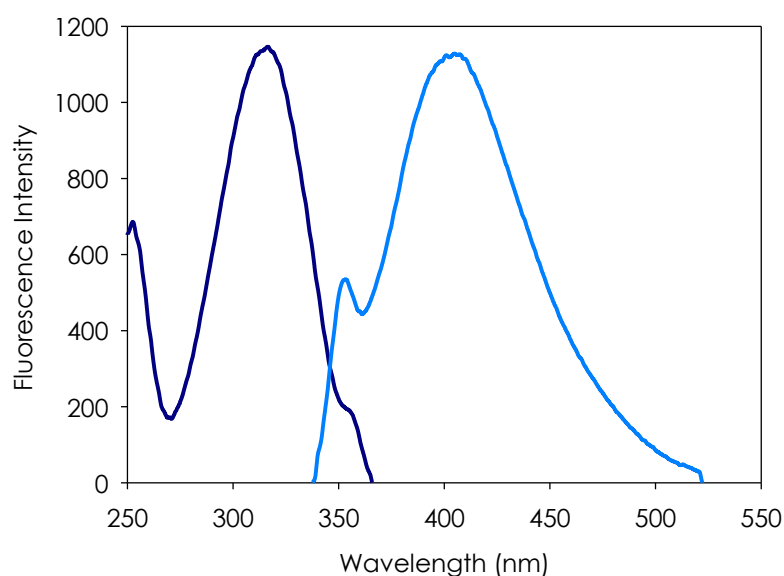


Fig 7: Cinnabarinic acid emission and excitation fluorescence spectra recorded.

3.4 Fluorescence of anthranilic, 3-hydroxyanthranilic and cinnabarinic acids with different pH

As in absorbance, pH have a high influence in the fluorescence intensity of the compounds, as can be seen in Fig 8 anthranilic acid fluorescence was higher at mild acid pH (3.0-4.0), whilst 3-hydroxyanthranilic acid was more fluorescent at neutral slightly basic pH (7.0-8.0), the only difference between the structures was the hydroxyl group in position 3 and was supposed to be responsible of the shift in the optimal pH for fluorescence. The fluorescent intensity increased around four times when pH was fixed in its optimum, note that the excitation and emission wavelengths were fixed in 360/460, so is possible that the fluorescence spectra changes with the changes of pH and the maximum peak emission and excitation wavelengths change as happen with the absorbance, however the pH study was make at fixed wavelength to assess the compounds as possible fluorophores for labeling.

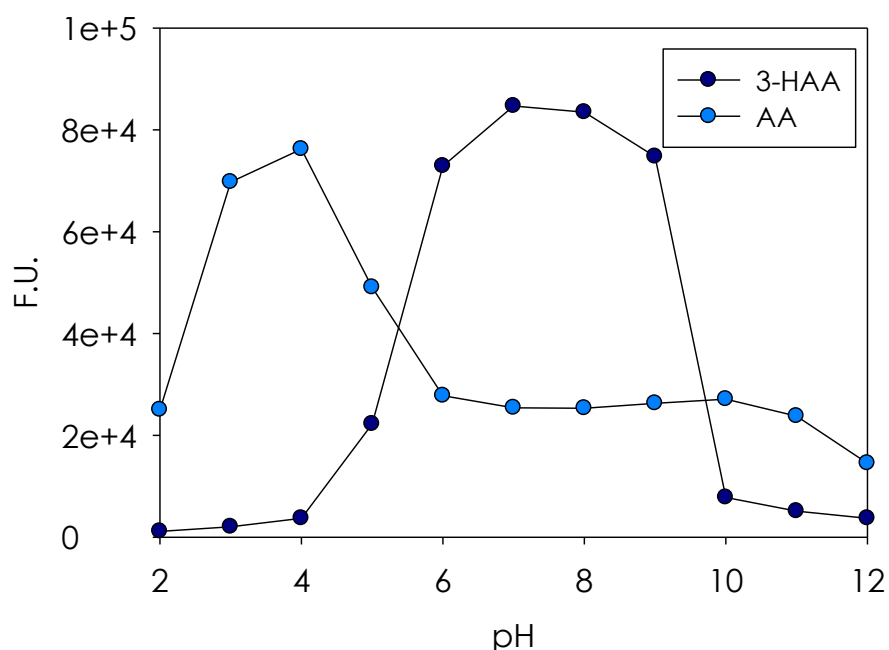


Fig 8: Relationship between pH and fluorescence intensity for anthranilic acid (●) and 3-Hydroxyanthranilic acid (●).

Unlike the other compounds cinnabarinic acid higher fluorescence intensity was obtained at basic pH (10.0-12.0) as showed Fig 9, as commented above the fluorescence intensity was low when compared with anthranilic and 3-hydroxyanthranilic acids and the compound gets slightly degraded with incident light.

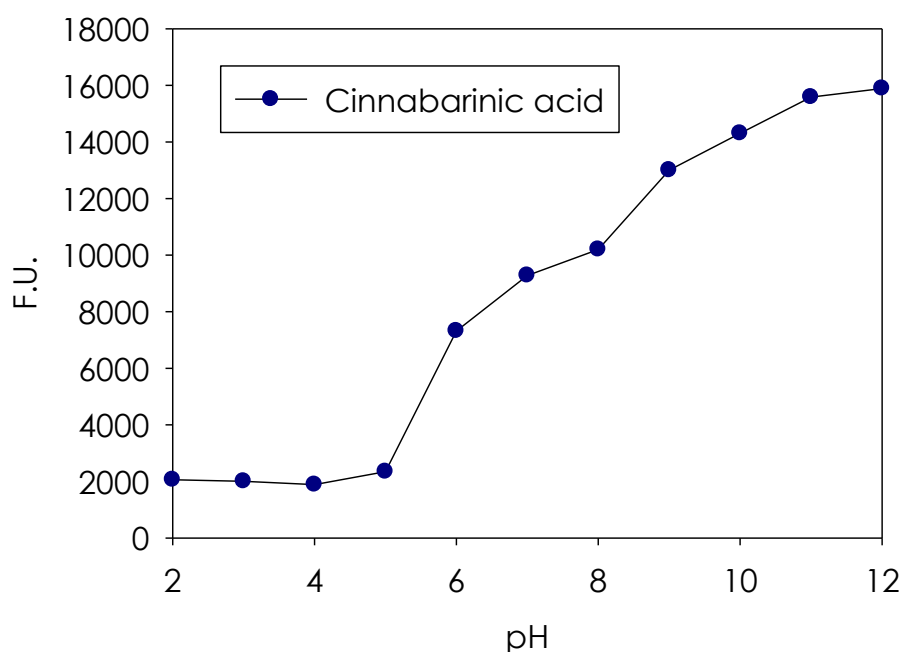


Fig 9: Relationship between pH and fluorescence intensity for Cinnabarinic acid.

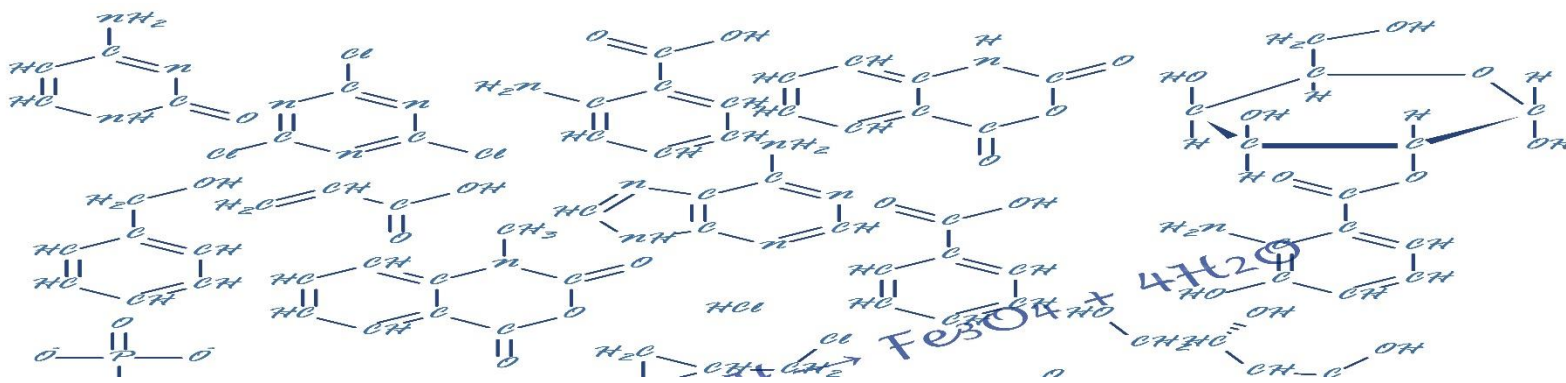
4 Conclusions

Anthranilic, 3-hydroxyanthranilic, and cinnabaric acids had been spectrophotometrically characterized, the results showed that the compounds absorbance and fluorescence intensity were strongly related to pH. Fluorescence studies had shown that cinnabaric acid had low fluorescence with an excitation maximum in 330 nm and an emission maximum of 404 nm. Water affinity had shown that cinnabaric acid had more affinity toward water molecules than its precursor and anthranilic acid with an affinity constant three times lower.

5 References

- Abou-Zied, O.K., Al-Busaidi, B.Y. & Husband, J., 2013. Solvent effect on anthranilic acid spectroscopy. *The Journal of Physical Chemistry A*, 118(1), pp.103–109.
- Bigge, J.C. et al., 1995. Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. *Analytical biochemistry*, 230(2), pp.229–238.
- Britton, H.T.S., 1932. Hydrogen Ions: Their Determination and Importance in Pure and Industrial Chemistry, Van Nostrand.
- Coburn, C. et al., 2013. Anthranilate fluorescence marks a calcium-propagated necrotic wave that promotes organismal death in *C. elegans*. *PLoS Biol*, 11(7), p.e1001613.
- Darlington, L.G. et al., 2010. On the biological importance of the 3-hydroxyanthranilic acid: anthranilic acid ratio. *International Journal of Tryptophan Research*, 3, p.51.
- Fazio, F. et al., 2012. Cinnabarinic acid, an endogenous metabolite of the kynurenine pathway, activates type 4 metabotropic glutamate receptors. *Molecular pharmacology*, 81(5), pp.643–656.
- Gems, D. & Coburn, C., 2013. The mysterious case of the *C. elegans* gut granule: death fluorescence, anthranilic acid and the kynurenine pathway. *Frontiers in genetics*, 4, p.151.
- Hill, T.L., 2013. *Cooperativity theory in biochemistry: steady-state and equilibrium systems*, Springer Science & Business Media.
- McMillan, M., 1960. The identification of a fluorescent reducing substance in the urine of patients with rheumatoid arthritis the excretion of 3-hydroxyanthranilic acid in this and other conditions. *Journal of clinical pathology*, 13(2), pp.140–148.

Chapter 14



Conclusions

Conclusions

The results obtained in this Thesis permitted to establish the following conclusions:

1 Replacing the glutaraldehyde reagent with epoxy reagents improved the carrier free immobilization method CLEAs.

- 1.1 The epoxy reagents tested in the assays produced active and stable epoxy-CLEAs.
- 1.2 The activity of the prepared epoxy-CLEAs was optimized using a mathematical approach, the design of experiments and the response surface. Using these approach epoxy-CLEAs with a 98 % activity yield were obtained.
- 1.3 The incorporation of magnetic nanoparticles to the production method, allowed obtaining of epoxy-CLEAs with magnetic properties, which were active and stable but can be removed from the solution with a neodymium magnet.

2 Siliceous mesocellular foam is a robust and stable support to immobilize laccase to be used in oxidation reactions.

- 2.1 Therefore, the laccase immobilization inside the (SMC foam) pores using a triepoxide compound was optimized using a Box-Behnken model. The optimized model retain a 90 % activity. The optimized biocatalyst was used to prepare two phenoxazinones, the Curie-22 and cinnabarinic acid. In both cases the biocatalyst maintain the activity yield in ten cycles of reuse.

3 A method to prepare magnetic enzymes was developed and applied to a lipase.

- 3.1 Employing the hydroxyl groups presented in the magnetic nanoparticles surface and organic halides, magnetic nanoparticles functionalized can be produced. With this procedure the particles maintained its nanometric size, magnetic properties and the oxidation was avoided.

- 3.2 A *P. fluorescens* lipase was attached to magnetic particles functionalized with epoxy and cyanogen. The biocatalyst bond with epoxides showed better performance than the cyanogen one.
- 3.3 The lipase attached to the support had 110 % activity yield, showing that the hydrophobic surface of the magnetic particles promoted the lipase superactivation. The biocatalyst can be implemented in a solvent free reaction with good results. Benzyl acetate solvent free synthesis
- 3.4 Using an economically affordable co-solvent, ethylene glycol, and hydrophobic p-nitrophenyl esters as substrates lipase hydrolytic activity could be measured in aqueous buffer. It had been demonstrated that the co-solvent does not interfere in the measurements, but does not interact with the released chromophore, Ca²⁺ ions or buffers. The boiling point of the compound enables activity to be measured at highest temperatures, which is not possible with other organic solvents as they evaporate.

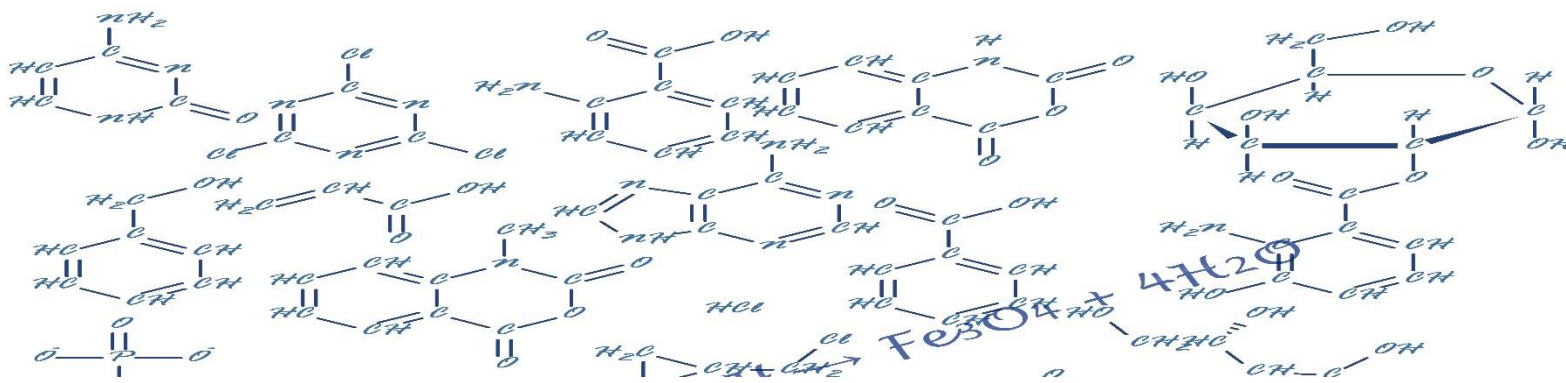
4 N-methylisatoic anhydride and aminofluorescein fluorescent derivatives had been produced and exploited as protein labeling reagents, acrylamide gels staining dyes and cell signaling reagents.

- 4.1 A method to prepare reactive fluorescein had been developed, using aminofluorescein and cyanuric chloride the triazine activated fluorescein can be produced. This reagent was able to react with protein side chain amines and primary amines.
- 4.2 N-methylisatoic anhydride can be used to covalent label proteins with fluorescence.
- 4.3 By reaction with the metal chelator (NTA), HIS-tag proteins can be selectively labeled in acrylamide gels, with a detection limit of 50 ng of HIS-tag protein per band.

Chapter 14

- 4.4 Using fluorescent polarization measurement the affinity constant of the NTA-fluoropores with HIS-tag proteins can be calculated.
- 4.5 By the reaction of this fluorophores to stearylamine, hydrophobic fluorescent reagents were produced. The reagents were successfully applied to gel stain and cell membrane detection. Also a fluorescent protein quantification in liquid media was proposed. The results showed more sensitivity than Bradford reagent.
- 4.6 The confocal images of the cells showed that the fluorophore only stained the membrane and did not penetrate to the cytoplasm.

Chapter 15



References

Chapter 15

Abdel-Naby, M.A. et al., 1998. Production and immobilization of alkaline protease from *Bacillus mycoides*. *Bioresource Technology*, 64(3), pp.205–210.

Ahmed, H., 2004. *Principles and reactions of protein extraction, purification, and characterization*, CRC press.

Andreescu, S., Barthelmebs, L. & Marty, J.-L., 2002. Immobilization of acetylcholinesterase on screen-printed electrodes: comparative study between three immobilization methods and applications to the detection of organophosphorus insecticides. *Analytica Chimica Acta*, 464(2), pp.171–180.

Anwar, A. et al., 2009. Calcium alginate: a support material for immobilization of proteases from newly isolated strain of *Bacillus subtilis* KIBGE-HAS. *World Applied Sciences Journal*, 7(10), pp.1281–1286.

Asherie, N. et al., 1998. Oligomerization and phase separation in globular protein solutions. *Biophysical chemistry*, 75(3), pp.213–227.

Baek, W.-O. & Vijayalakshmi, M.A., 1997. Effect of chemical glycosylation of RNase A on the protein stability and surface histidines accessibility in immobilized metal ion affinity electrophoresis (IMAGE) system. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1336(3), pp.394–402.

Bilková, Z. et al., 2002. Enzymes immobilized on magnetic carriers: efficient and selective system for protein modification. *Journal of Chromatography B*, 770(1), pp.177–181.

Brady, D. et al., 2008. Spherezymes: A novel structured self-immobilisation enzyme technology. *BMC biotechnology*, 8(1), p.8.

Cormier, M.J., 1992. Recombinant DNA vectors capable of expressing apoaequorin in *E. coli* U.S. Patent No. 5,162,227.

Chapter 15

Cristóvão, R.O. et al., 2011. Immobilization of commercial laccase onto green coconut fiber by adsorption and its application for reactive textile dyes degradation. *Journal of Molecular Catalysis B: Enzymatic*, 72(1), pp.6–12.

Cronan, J.E., 1990. Biotination of proteins in vivo. A post-translational modification to label, purify, and study proteins. *Journal of Biological Chemistry*, 265(18), pp.10327–10333.

van Dam, A. et al., 2014. Immunolabeling of fingerprints left on forensic relevant surfaces, including thermal paper. *Analytical Methods*, 6(4), pp.1051–1058.

Datta, S., Christena, L.R. & Rajaram, Y.R.S., 2013. Enzyme immobilization: an overview on techniques and support materials. *3 Biotech*, 3(1), pp.1–9.

Davies, G.E. & Stark, G.R., 1970. Use of dimethyl suberimidate, a cross-linking reagent, in studying the subunit structure of oligomeric proteins. *Proceedings of the National Academy of Sciences*, 66(3), pp.651–656.

DeSantis, G. & Jones, J.B., 1999. Chemical modification of enzymes for enhanced functionality. *Current Opinion in Biotechnology*, 10(4), pp.324–330.

Dutta, P.K., Dutta, J. & Tripathi, V.S., 2004. Chitin and chitosan: Chemistry, properties and applications. *Journal of Scientific & Industrial Research*, 63, 20-31.

Giepmans, B.N.G. et al., 2006. The fluorescent toolbox for assessing protein location and function. *science*, 312(5771), pp.217–224.

De Groot, A.S. & Scott, D.W., 2007. Immunogenicity of protein therapeutics. *Trends in immunology*, 28(11), pp.482–490.

Halling, P.J. & Dunnill, P., 1980. Magnetic supports for immobilized enzymes and bioaffinity adsorbents. *Enzyme and Microbial Technology*, 2(1), pp.2–10.

Chapter 15

Hansen, A.S. & O'shea, E.K., 2013. Promoter decoding of transcription factor dynamics involves a trade-off between noise and control of gene expression. *Molecular systems biology*, 9(1), p.704.

Hernaiz, M.J., Sánchez-Montero, J.M. & Sinisterra, J. V, 1997. Influence of the nature of modifier in the enzymatic activity of chemical modified semipurified lipase from *Candida rugosa*. *Biotechnology and bioengineering*, 55(2), pp.252–260.

Hernández-García, S., García-García, M.I. & García-Carmona, F., 2014. Purification, Immobilization and Characterization of Lipase Isoenzyme from *Aspergillus niger* with C8 Magnetic Particles. *Advances in Bioscience and Biotechnology*, 5(7), p.633.

Hoshi, T., Anzai, J. & Osa, T., 1995. Controlled deposition of glucose oxidase on platinum electrode based on an avidin/biotin system for the regulation of output current of glucose sensors. *Analytical chemistry*, 67(4), pp.770–774.

Hyoudou, K. et al., 2006. PEGylated catalase prevents metastatic tumor growth aggravated by tumor removal. *Free Radical Biology and Medicine*, 41(9), pp.1449–1458.

Krajewska, B., 2004. Application of chitin-and chitosan-based materials for enzyme immobilizations: a review. *Enzyme and microbial technology*, 35(2), pp.126–139.

Leary, J.J., Brigati, D.J. & Ward, D.C., 1983. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. *Proceedings of the National Academy of Sciences*, 80(13), pp.4045–4049.

Liu, B., Hu, R. & Deng, J., 1997. Characterization of immobilization of an enzyme in a modified Y zeolite matrix and its application to an amperometric glucose biosensor. *Analytical chemistry*, 69(13), pp.2343–2348.

Chapter 15

Mateo, C. et al., 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and microbial technology*, 40(6), pp.1451–1463.

Means, G.E. & Feeney, R.E., 1990. Chemical modifications of proteins: history and applications. *Bioconjugate chemistry*, 1(1), pp.2–12.

Mena, M.A. et al., 2006. Blue fluorescent proteins with enhanced brightness and photostability from a structurally targeted library. *Nature biotechnology*, 24(12), pp.1569–1571.

Persechini, A. & Kretsinger, R.H., 1988. The central helix of calmodulin functions as a flexible tether. *Journal of Biological Chemistry*, 263(25), pp.12175–12178.

Quioco, F.A. & Richards, F.M., 1964. Intermolecular cross linking of a protein in the crystalline state: carboxypeptidase-A. *Proceedings of the National Academy of Sciences*, 52(3), pp.833–839.

Schlieker, M. & Vorlop, K.-D., 2006. A novel immobilization method for entrapment: LentiKats®. *Immobilization of enzymes and cells*, pp.333–343.

Serralha, F.N. et al., 1998. Zeolites as supports for an enzymatic alcoholysis reaction. *Journal of Molecular Catalysis B: Enzymatic*, 4(5), pp.303–311.

Sheldon, R.A., 2011. Characteristic features and biotechnological applications of cross-linked enzyme aggregates (CLEAs). *Applied microbiology and biotechnology*, 92(3), pp.467–477.

Sheldon, R.A., Schoevaart, R. & van Langen, L.M., 2003. CLEAs: An effective technique for enzyme immobilisation. *Speciality chemicals*, 23(6), pp.40–42.

Shimomura, O., Johnson, F.H. & Saiga, Y., 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusa, *Aequorea*. *Journal of cellular and comparative physiology*, 59(3), pp.223–239.

Chapter 15

Silva, C. et al., 2004. Chemical modifications on proteins using glutaraldehyde. *Food Technology and Biotechnology*, 42(1), pp.51–56.

Streitwieser, A. et al., 1992. *Introduction to organic chemistry*, Macmillan New York.

Tinoco, R. & Vazquez-Duhalt, R., 1998. Chemical modification of cytochrome C improves their catalytic properties in oxidation of polycyclic aromatic hydrocarbons. *Enzyme and microbial technology*, 22(1), pp.8–12.

Uchiumi, T. et al., 1985. Cross-linking study on protein topography of rat liver 60 S ribosomal subunits with 2-iminothiolane. *Journal of Biological Chemistry*, 260(9), pp.5675–5682.

Wilson, N.G., McCreedy, T. & Greenway, G.M., 2000. In-situ immobilisation of glucose oxidase on a novel microporous silica support. *Analyst*, 125(2), pp.237–239.

RESUMEN

Esta tesis doctoral titulada "Modificación química de proteínas, aplicaciones en inmovilización, actividad catalítica y marcaje fluorescente" desarrolla metodologías de modificación de proteínas y aplicaciones de las mismas. Se ha dividido en cuatro bloques en cada uno de los cuales se aborda una metodología de modificación de proteínas y se propone una o más aplicaciones de la metodología.

En un primer bloque de contenido se plantea mejorar la tecnología de inmovilización "sin soporte": CLEAs (cross-linked enzyme aggregates). La metodología de CLEAs tiene un primer paso en el que las proteínas son precipitadas usando altas concentraciones de sales, como por ejemplo sulfato amónico, disolventes orgánicos: etanol, propanol o acetona o polímeros como los poli-etilenglicoles. Una vez precipitadas las proteínas, estas se unen entre si empleando glutaraldehído, la reacción que tiene lugar es la formación de una base de Schiff entre las aminas de las cadenas laterales de las proteínas y los grupos aldehído del reactivo. La bases de Schiff es un equilibrio que puede romperse al variar el pH de disolución (con ácidos o bases) liberando proteínas al medio de reacción. Teniendo esto en cuenta se propone reemplazar el glutaraldehído por compuestos diepóxido. Ya que los epóxidos reaccionan con aminas y se obtienen aminas secundarias, este enlace es covalente e irreversible. Esta sustitución evita la contaminación del producto final y la pérdida de enzimas. También mejora la fabricación del biocatalizador, mientras que la base de Schiff se favorece a pH ligeramente ácido, la reacción de epóxidos se puede ajustar al pH óptimo de la enzima, asegurando su estabilidad estructural y rendimiento catalítico.

El método se ha implantado con éxito en la producción de agregados reticulados con aldolasa (CLEAs) y también en la producción de CLEAs magnéticas de aldolasa que incluyen la incorporación de nanopartículas magnéticas (9-12 nm) a agregados enzimáticos magnéticos. Las CLEAs de aldolasa obtenidas conservan un 98% de rendimiento. La incorporación de partículas magnéticas, mejora las posibilidades de uso de estas preparaciones enzimáticas en procesos industriales como facilidad de manejo del catalizador. La posibilidad de separar fácilmente el catalizador del medio de reacción, empleando un imán de neodimio, facilita su extracción y reutilización,

evita la contaminación de los productos finales y evita costosas etapas de centrifugación. Las epoxi-CLEAs magnéticos producen un 80 % de rendimiento de actividad, mostrando la versatilidad de los reactivos de cross-linking propuestos.

En el segundo bloque la lacasa de *Trametes versicolor* es inmovilizada sobre o dentro de soportes de sílice, tanto comerciales como sintetizados en el laboratorio. Los resultados de estas pruebas preliminares dieron como mejor soporte para la enzima los soportes de sílice mesoporosa sintetizados en el laboratorio. Por tanto, se ha inmovilizado la enzima lacasa dentro de los poros de un soporte preparado de sílice mesocelular, la enzima se ha unido a los poros de soporte, funcionalizados con grupos amino mediante un compuesto triepóxido. El epóxido reacciona con los grupos amina tanto de la enzima como del soporte y ancla la lacasa a los poros de soporte. Esta es la primera vez que el glutaraldehído (el reactivo de cross-linking más utilizado en inmovilización) ha sido reemplazado por un poliepóxido. Utilizando un diseño experimental Box-Behnken, se optimizaron las variables de inmovilización, alcanzando un rendimiento de actividad del 90 % en comparación con la enzima libre. El biocatalizador optimizado se ensayó en la formación de acoplamiento de oxidación de un colorante de fenoxazinona. Sus propiedades incluyeron resistencia a la temperatura y al pH, fuerte afinidad por el sustrato 3-amino-4-hidroxibenceno sulfonato (K_M 72 μ M) y buena reutilización (diez ciclos se pueden realizar con la mínima pérdida de rendimiento) cuando el proceso se escaló. En resumen, este biocatalizador puede considerarse económicamente viable para reacciones de oxidación, todavía realizadas con oxidantes como el periodato, o el permanganato.

Empleando el mismo biocatalizador se propuso estudiar la síntesis del ácido cinabarínico, ya que ha recibido mucha atención durante las últimas décadas en estudios clínicos. Debido a que su precursor el ácido 3-hidroxiantranílico es producido por interferón- γ fagocitos mononucleares y parece estar implicado en la protección de los tejidos de mamíferos contra las especies de oxígeno reactivas. Recientemente el ácido cinabarínico también se ha estudiado como compuesto farmacéutico para enfermedades autoinmunes, neurodegenerativas y otros trastornos neurológicos, así como con enfermedades psiquiátricas. Este nuevo interés ha promovido la aplicación del biocatalizador de lacasa para la obtención sostenible del ácido cinabarínico. El sustrato y el producto se han caracterizado espectrofotométricamente, y se ha

propuesto el mecanismo de reacción para la oxidación acoplada. El biocatalizador de lacasa tiene un 80 % de rendimiento para la formación de ácido cinabárnico y presenta buenas propiedades: buena afinidad para el sustrato, ya que tiene un K_M inferior ($25 \mu\text{M}$) a la de la enzima libre, buena reutilización (diez ciclos de recuperación de la producción sin pérdida de actividad) y buena selectividad porque no se producen subproductos (detectados por análisis HPLC).

En un tercer bloque se propone la modificación directa de nanopartículas magnéticas (9-12 nm). Estas partículas son funcionalizadas directamente por sustitución nucleofílica con haluros orgánicos. Los halogenuros son compuestos orgánicos que contienen uno o más grupos halógenos, tales como las epiclorhidrina, la 1, 3, 5 triclora 2, 4, 6 triazina, el bromuro y cloruro de cianógeno, el cloruro de p-toluenosulfonilo u otros haluros orgánicos capaces de reaccionar con grupos hidroxilo. El método propuesto mejora el procedimiento general de funcionalización de partículas magnéticas, que incluye un revestimiento polimérico sobre las partículas magnéticas y luego el proceso de funcionalización. El recubrimiento de polímero tiende a actuar como un escudo inerte que disminuye la respuesta de las partículas a los campos magnéticos. Nuestro método de modificación directa en la superficie de la partícula evita este fenómeno, además no aumenta el tamaño de las partículas y es económicamente sostenible.

Las nanopartículas magnéticas obtenidas, funcionalizadas con este método, tienen grupos reactivos como grupos cianato, cloro reactivo, epoxy o tosilo disponibles para reaccionar con moléculas biológicas, como proteínas, enzimas, ácidos nucleicos o anticuerpos y los unen covalentemente a la superficie magnética. También podría ser modificado químicamente, añadiendo grupos reactivos tales como aminos orgánicos, se podría cambiar la naturaleza de las partículas, haciéndolas más hidrófilas (cargas positivas o negativas) o hidrófobas.

Usando las nanopartículas funcionalizadas se propone un método sostenible para obtener un acetato de bencilo seminatural en un sistema libre de disolvente. Se preparó un biocatalizador de lipasa magnético nanométrico (15 nm). Se prepararon nanopartículas magnéticas modificadas con grupos epoxy y ciano en la superficie, a continuación, la enzima lipasa es unida covalentemente a las partículas obteniendo un biocatalizador de tamaño nanométrico.

La síntesis de acetato de bencilo es estudiada, se han optimizado las cantidades de sustrato (acetato de vinilo y alcohol bencílico), la cantidad de agua y temperatura, también se estudian los parámetros cinéticos. Con el biocatalizador nanométrico se obtiene una conversión del 90 % en 6 horas con una cantidad máxima de 300 g de acetato de bencilo producido por litro. El catalizador magnético permite una fácil separación y recuperación de la enzima usando un imán, obteniendo un producto final libre de enzima.

Durante este bloque nos surge la necesidad de tener un método sencillo y directo de medida de actividad de lipasa, lo que nos llevó a optimizar la medida de actividad hidrolítica de lipasa con p-nitrofenil acil-ésteres. Se propone un método mejorado basado en el método del palmitato de p-nitrofenilo para medir la actividad hidrolítica de la lipasa en medios acuosos. Usando etilenglicol como co-disolvente para los sustratos hidrófobos de p-nitrofenilo en tampón acuoso, la actividad de la lipasa es medida siguiendo la liberación de p-nitrofenol (un compuesto de color amarillo) a 405 nm. El método mejora la solubilidad del sustrato y del producto, también la estabilidad del sustrato (en etilenglicol el sustrato no se hidroliza), evita el uso de disolventes orgánicos (etanol, metanol o hexano) que pueden inhibir la enzima y son difíciles de manejar. Permite la comparación de todos los sustratos derivados del p-nitrofenol, permite el estudio de tensioactivos sobre las enzimas, y la influencia de iones como Ca^{+2} puede estudiarse sin turbidez en el medio. El método propuesto es rápido, fácil de manejar y lineal en un amplio rango de concentración de enzima de 0-0.8 mg / mL. Para comprobar la utilidad del método se usaron dos lipasas obtenidas de diferentes organismos *Pseudomona fluorescens* y *Mucor javanicus*.

El cuarto y último bloque es la aplicación de derivados fluorescentes sintetizados en el laboratorio para aplicarlos en biología molecular y celular: marcaje y detección de proteínas, cuantificación de proteínas o marcaje celular.

Se propone un método de marcaje de proteínas en medio líquidos y una técnica de teñido geles de electroforesis de acrilamida con fluorescencia. Utilizando fluoresceína activada con triazina sintetizada en nuestro laboratorio y anhídrido metilisatoico, un compuesto fluorescente disponible comercialmente. Las proteínas se marcaron covalentemente con los fluoróforos y se sometieron a electroforesis en geles de acrilamida o los geles de acrilamida se tiñeron directamente con los fluoróforos. Los

resultados mostraron que la triazina fluoresceína activada y el anhídrido n-metilatoico son capaces de marcar las proteínas en medio líquido y teñir los geles de acrilamida. La fluoresceína activada ha mostrado tanta sensibilidad como el teñido de rutina: azul brillante coomassie.

La amino fluoresceína activada es dos veces más intensa que el metilatoico y casi tan sensible como el procedimiento de tinción normal (azul coomassie coloidal). Sin embargo anhídrido metilatoico es más barato (100 g cuesta 65.90 €) que la fluoresceína activada con triazina (5 g cuesta 381.50 €). Sin embargo, estos precios son realmente económicos en comparación con los colorantes fluorescentes disponibles en el mercado como la fluorescamina (1 g cuesta 607.0 €) o verde indocyanina (250 mg cuesta 333.60 €). Por lo tanto, el bajo coste de estos fluoróforos reactivos permite su incorporación al procedimiento de trabajo cotidiano como marcaje de proteínas o tinción de gel.

Se han sintetizado dos sondas fluorescentes utilizando el anhídrido n-metilatoico y la aminofluoresceína como fluoróforos y el quelato de metales, el ácido nitrilotriacético (NTA) para producir marcadores de afinidad fluorescentes, aplicados para detectar específicamente la etiqueta de oligo-histidina de las proteínas sobreexpresadas. Las sondas se usaron para detectar proteínas HIS-tag en un gel de electroforesis de acrilamida, el método puede detectar tan poco como 50 ng de proteína. Después, las sondas se aplicaron con éxito en medidas de anisotropía de fluorescencia de unión de un receptor (proteína HIS-tag) al ligando (metilatoico-NTA).

Los fluoróforos-NTA sintetizados han resultado ser económicamente viables para emplearlos en la rutina diaria de laboratorio, el precio estimado para teñir un mini gel es de alrededor de 0.13-0.26 €, mientras que la tinción de un mini gel con NTA comercial fluorescente conjugado cuesta alrededor de 29 €.

Siguiendo la misma línea, se han sintetizado dos fluoróforos hidrofóbicos uniendo una cola hidrofóbica de octadecilo (una cadena compuesta por dieciocho alquilos) al anhídrido n-metilatoico y a la aminofluoresceína activada con triazina. Debido a la cola hidrofóbica los fluoróforos sintetizados se unen no covalentemente a proteínas desnaturalizadas con SDS, esta característica se aplica en el teñido de geles y a la señalización celular. También se ha propuesto un método fluorescente para

cuantificar proteínas en medio líquido utilizando el n-metilisatoico C18 sintetizado. El compuesto mostró un aumento de fluorescencia proporcional a la cantidad de proteína añadida, el método es lineal entre 0.02 y 1 mg / mL de proteína, resultando más sensible que el método Bradford. La cola hidrofóbica también se une a otras moléculas hidrofóbicas tales como los fosfolípidos de membrana, cola del fluoróforo se inserta entre dos fosfolípidos dejando el fluoróforo en el exterior de la membrana y permitiendo la visualización de células. Las bacterias Gram positivas *Staphylococcus carnosus* y de *Bacillus subtilis* han sido teñidas y visualizadas mediante microscopía confocal.

En términos de fluorescencia C18-FL tienen alrededor de 2,5 veces más intensidad de fluorescencia que C18-MI con la misma concentración de fluoróforo. Sin embargo, en términos de precio, el anhídrido n-metilisatoico (100 g cuesta 65.90 €) es notablemente más económico que la aminofluoresceína (5 g cuesta 381.50 €), convirtiendo al fluoróforo C18-MI en un reactivo económicamente viable para labores diarias como teñido de geles de acrilamida, cuantificación de proteínas y marcaje fluorescente de células.