Universitat Autònoma de Barcelona

Use of alcohol dehydrogenase and alcohol oxidase to convert alcohols in two valuable products: chlorolactone and vanillin

Miquel García Bofill

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Universitat Autònoma
de Barcelona

Escola d'Enginyeria

Departament d'Enginyeria Química, Biològica i Ambiental

Use of alcohol dehydrogenase and alcohol oxidase to convert alcohols in two valuable products: chlorolactone and vanillin

Memòria per optar al grau de Doctor per la Universitat Autònoma de Barcelona, sota la direcció de la Dra. Marina Guillén Montalbán, el Dr. Gregorio Álvaro Campos i el Dr. Peter W. Sutton

per

Miquel García Bofill

Bellaterra, Febrer 2021

La Dra. Marina Guillén Montalbán, professora del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona, El Dr. Gregorio Álvaro Campos, professor Titular del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona i el Dr. Peter Sutton, postdoc del Departament d'Enginyeria Química, Biològica i Ambiental.

Certifiquem:

Que l'enginyer de sistemes biològics Miquel García Bofill ha dut a terme sota la nostra direcció el treball titulat **Use of alcohol dehydrogenase and alcohol oxidase to convert alcohols into two valuable products: chlorolactone and vanillin**, que es presenta en aquesta memòria i constitueix la seva Tesis per optar al Grau de Doctor en Biotecnologia per la Universitat Autònoma de Barcelona.

I per tal que se'n prengui coneixement i consti als efectes oportuns, signem la present a Bellaterra, 15 Febrer de 2021.

Dra. Marina Guillén Montalbán Dr. Gregorio Álvaro Campos Dr. Peter W. Sutton

Resum

Els enzims presenten una sèrie d'avantatges catalítiques respecte als catalitzadors químics emprats en síntesis química clàssica: especificitat, selectivitat i la possibilitat de treballar en condicions suaus de temperatura i pressió. No obstant, també presenten una sèrie de limitacions com són la baixa estabilitat i les baixes productivitats. En el present treball es combinen dues tècniques per tractar d'optimitzar les reaccions d'interès seleccionades: la immobilització i l'enginyeria de reacció.

Les reaccions objectiu d'aquest treball són reaccions d'oxidoreducció centrades en la biosíntesis de molècules, de mitjà i alt valor afegit, d'alt interès industrial. En la primera part de la tesis s'ha utilitzat una alcohol deshidrogenasa (ADH99) per a la oxidació de l'alcohol chlorolactol a chlorolactona i una NAD(P)H oxidasa (NOX) com a sistema de regeneració del cofactor. La chlorolactona és un precursor per a la síntesis d'estatines les quals són fàrmacs utilitzats per a la reducció del LDL-colesterol ja que inhibeixen l'enzim encarregat de la seva biosíntesis. Ambdós enzims van ser immobilitzats eficientment en diferents suports, dels quals es van seleccionar els tres que van mostrar major activitat retinguda. Seguidament es va estudiar l'estabilitat dels derivats immobilitzats en condicions de reacció i es va determinar la càrrega enzimàtica màxim per a cada enzim. Es va descartar l'ús de la NOX immobilitzada ja que no es va millorar l'estabilitat amb cap suport. Posteriorment es van optimitzar les condicions de reacció amb un disseny experimental (DoE) amb l'ADH99 soluble però utilitzant la quantitat màxima d'ADH99 que es pot afegir a la reacció quan es fa servir el l'ADH99 immobilitzada en epoxy-agarosa-UAB M2. Finalment es va estudiar la capacitat de reutilització del derivat immobilitzat, on es va poder millorar 1.5 vegades tant el producte obtingut com el rendiment del biocatalitzador. No obstant, la millor configuració va resultar ser la utilització dels dos enzims en forma soluble.

La segona part d'aquesta tesis es va centrar en la reacció d'oxidació de l'alcohol vainillínic a vanil·lina biocatalitzada per l'eugenol oxidasa (EUGO). La vanil·lina és la molècula que dona les propietats organolèptiques a la vainilla, el segon aromatitzant més car del món. La síntesi

de vanil·lina via biotecnològica és d'un gran interès industrial ja que el producte obtingut pot etiquetar-se com a natural. De manera similar a l'anterior apartat, l'EUGO va ser immobilitzada eficientment en diferents suports dels que es van seleccionar els tres que van retenir més activitat. Aquests es van utilitzar per estudiar l'estabilitat de l'enzim immobilitzat i la màxima carrega enzimàtica que se'n pot immobilitzar. En aquest cas els tres derivats immobilitzats van ser utilitzats per a realitzar la reacció de síntesi, amb l'objectiu de seleccionar el més estable operacionalment. Tots els derivats van permetre ser reutilitzats 5 vegades conservant una elevada conversió en l'últim cicle. L'epoxy-agarosa-UAB M2 va ser el suport que millor estabilitat va mostrar, millorant el rendiment del biocatalitzador 3 vegades.

Els bons resultats obtinguts en el segon apartat d'aquest treball van permetre aprofundir en aquesta reacció. Pel que, en el tercer apartat, es va realitzar una optimització de les condicions de reacció des del punt de vista de millorar les mètriques del procés i també amb l'objectiu de fer el procés més sostenible ambientalment. A l'hora d'escollir les noves condicions de reacció es van tenir en compte l'activitat de la EUGO i la seva estabilitat. Ambdues condicions van ser testades en la reacció objectiu amb l'EUGO soluble i immobilitzada. En les noves condicions es va poder millorar la productivitat volumètrica 5.7 i 6.6 vegades respectivament, en comparació a les condicions prèvies. Finalment, en el reciclatge de l'enzim immobilitzat es van poder realitzar 5 cicles de reacció en les primeres condicions i 18 cicles de reacció en les noves condicions on es va poder millorar el rendiment del biocatalitzador 3.9 i 12.4 vegades respectivament, en comparació a les reaccions realitzades amb enzim soluble en les mateixes condicions.

Abstract

Enzymes have some catalytic advantages over chemical catalysts used in classical chemical synthesis: specificity, selectivity and the possibility to work under mild conditions of temperature and pressure. However, they also have some limitations such as low stability and low productivity. This work combines two techniques aiming to optimise the target reactions: immobilisation and reaction engineering.

The target reactions of this work are redox reactions focused on the biosynthesis of molecules, of medium-high value, of industrial interest. In the first part of the thesis, an alcohol dehydrogenase (ADH99) was used, with an NAD(P)H oxidase (NOX) as a cofactor regeneration system, to oxidise a chlorolactol to chlorolactone. Chlorolactone is a precursor for the synthesis of statins which are drugs used to lower LDL-cholesterol by inhibiting the enzyme responsible for its biosynthesis. Both enzymes were efficiently immobilised on different supports, selecting the three that showed the highest retained activity. The stability of the immobilised derivatives under reaction conditions was studied and the maximum enzyme load for each enzyme also was determined. The use of immobilised NOX was discarded because no stability improvements were achieved with any support. The reaction conditions were optimised by design of experiments (DoE), using soluble ADH99 added at maximum loading onto an epoxy-agarose support. Finally, the reusability of the immobilised enzyme was studied, where both the total product obtained and the biocatalyst yield could be improved 1.5-fold. However, the best configuration resulted from the use of the two enzymes in soluble form.

The second part of this thesis was focused on the oxidation reaction of vanillyl alcohol to vanillin catalysed by eugenol oxidase (EUGO). Vanillin is the molecule that gives vanilla its organoleptic properties. Vanillin biotechnological synthesis is of high interest industrially because it is the second most expensive flavouring in the world and the product can be labelled as natural. Similar to the previous section, EUGO was efficiently immobilised onto different supports, selecting the three that retained most activity. These supports were used to study the stability of the immobilised enzyme and the maximum EUGO load that can be immobilised.

In this case, the three immobilised derivatives were used to perform the target reaction, in order to select the most stable operationally. All immobilised derivatives could be reused 5 times maintaining a high conversion in the last cycle. Epoxy-agarose-UAB M2 was the support that showed the best stability, improving the biocatalyst yield 3-fold.

The encouraging results obtained in the second section of this work allowed us to deepen the study of this reaction. Therefore, in the third section, an optimisation of the reaction conditions was carried out to improve the process metrics and also aiming to make the process more environmentally sustainable. The EUGO activity and its stability were taken into account to choose the reaction conditions. Both conditions, maximum activity and maximum stability, were tested in the target reaction with soluble and immobilised EUGO. Using the new conditions, it was possible to improve the volumetric productivity 5.7 and 6.6-fold respectively, compared to the previous conditions. Finally, the reusability of the immobilised EUGO allowed us to perform 5 reaction cycles and 18 reaction cycles, with unoptimised and optimised reaction conditions respectively. This resulted in an improvement of the biocatalyst yield of 3.9 and 12.4-fold, respectively, compared to reactions with soluble enzyme under the same conditions.

Abbreviations

ADH: Alcohol dehydrogenase
ADH99: Alcohol dehydrogenase from C-LEcta
ANOVA: Analysis of variance
AOX: Alcohol oxidase
ATP: Adenosine triphosphate
B: Biocatalyst
BBD: Box-Behnken design
BVMO: Beayer-Villiger monooxygenase
BY: Biocatalyst yield
CDI: (N-(3-dimethylaminopropyl)-N-ethyl) carbodiimide
CFE: Cell free extract
CVDs: Cardiovascular diseases
DERA: 2-Deoxy-D-ribose-5-phosphate aldolase
DoE: Design of experiments
ε: molar extinction coefficient
E. coli: *Escherichia coli*
EC: Enzyme Commission
EUGO: Eugenol oxidase
FAD$^+$: Flavin adenine dinucleotide oxidized form
FID: Flame ionization detector
GC: Gas chromatograph
GDH: Glucose dehydrogenase
HPLC: High performance liquid chromatograph
IS: Internal standard
IY: Immobilisation yield
K_{cat}: Catalytic constant
K_M: Michaelis constant
LDL: Low-density lipoprotein
LOF: Lack of fit
NAD$^+$: Nicotinamide adenine dinucleotide oxidated form
NADH: Nicotinamide adenine dinucleotide reduced form
NADP$^+$: Nicotinamide adenine dinucleotide phosphate oxidated form
NADPH: Nicotinamide adenine dinucleotide phosphate reduced form
NMR: Nuclear magnetic resonance
NOX: NADPH oxidase
P: Product
P450: cytochrome P450
PCR: Polymerase chain reaction
pI: Isoelectric point
r: Ratio
RA: Retained activity
ROBOX: European Union Project, grant agreement n° 635734. It stands for "Expanding the industrial use of Robust Oxidative Biocatalysts for the conversion and produc-

tion of alcohols”

rp: Initial product formation rate

S: Substrate

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

STY: Space-time yield

t: Time

$t_{1/2}$: Half-life

TFA: Trifluoroacetic acid

U: unit of activity

WP: Work package

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NOX I2 pH6
MIQUEL
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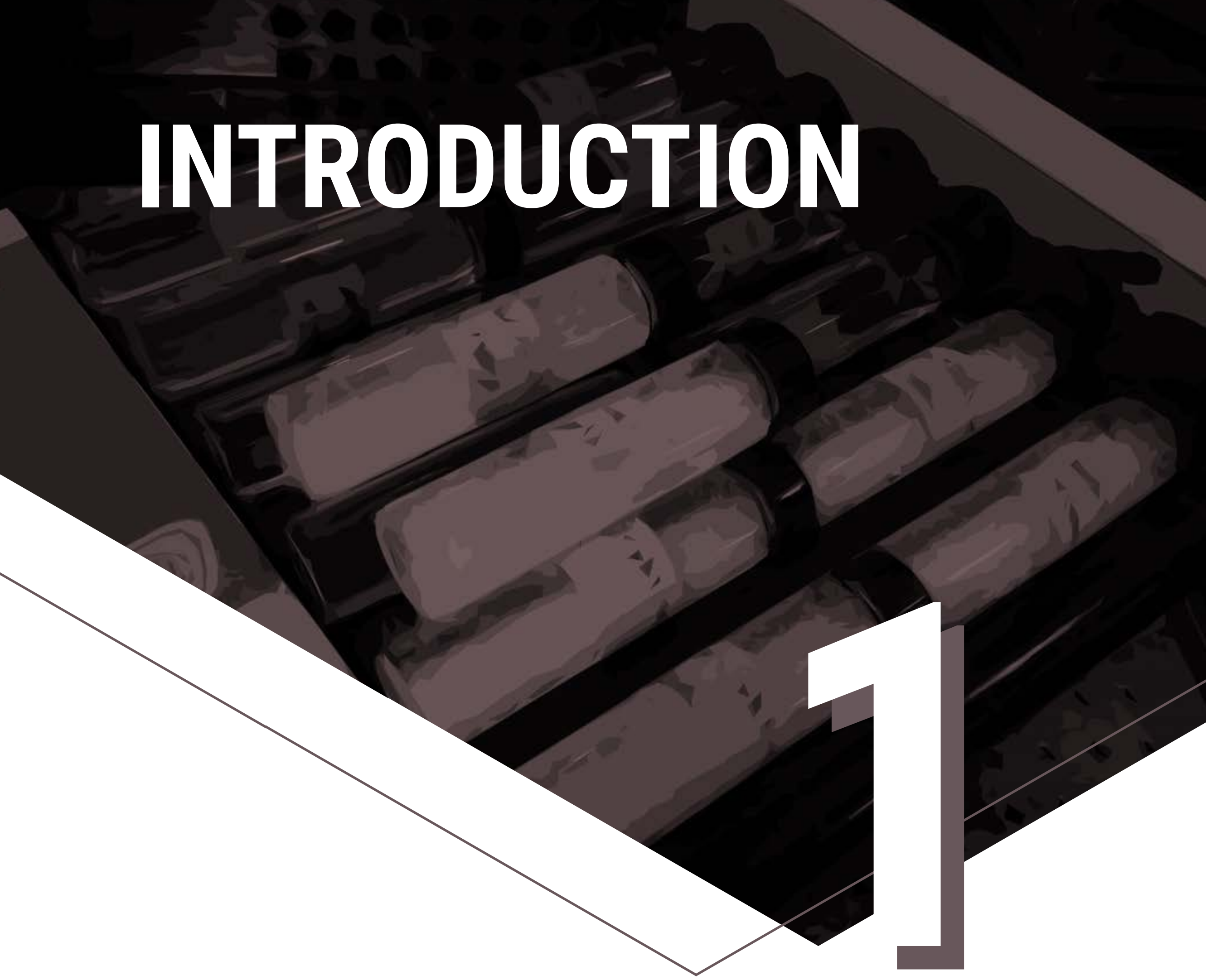
NOX I1 pH7

INTRODUCTION

1



1.1 Enzymes

In every living cell, even in the simplest one, thousands of different metabolic reactions occur. Practically each cellular reaction is catalysed by a different specific enzyme, which selectively generates a specific product. Enzymes catalyse these reactions by reducing the energy necessary to convert a molecule (substrate) to another (product), increasing the reaction rate more than 10^{12}-fold relative to the uncatalysed reaction rate. The enzyme interacts with the substrate forming an enzyme-substrate complex, triggering product formation. As with all catalysts, enzymes return to their initial form after substrate transformation, theoretically without alteration, ready to convert another molecule. Each molecule of enzyme can catalyse up to thousands of their natural reactions per second and the optimum reaction conditions of enzymes usually are under physiological conditions, since these are the conditions where life occurs. That implies that there are thousands of different enzymes that we can use in native form or modified genetically, as whole cells or purified, free or immobilised, to perform thousands of different specific reaction^{s} efficiently [1], [2]. Biocatalysis is considered greener and environmentally friendlier than chemical synthesis since it works at moderated temperature and pressure conditions, frequently the presence of solvents is avoided, and triggering the reduction of energy and costs related to the process. This aspect is also important and it is growing in interest since public concern about the environment is rising [3]. These excellent characteristics of enzymes make biocatalysts an exceptional tool to apply in the pharma, fine chemical and food industries, among others [4].

Nevertheless, enzymes have some drawbacks. Almost all enzymes are proteins which can be costly to produce and labile because their catalytic activity depends on their complex structure. The amino acids of these proteins interact forming four structural levels triggering complex three-dimensional structures, where the active centre is the specific region of the enzyme where the substrate is fixed and catalysis occurs. Evolutionarily, these complex structures have been adapted for working under physiological conditions. If we want to leverage the excellent properties of enzymes to perform industrial reactions, they need to work under more challenging conditions. Adverse conditions of temperature, pH, much high substrate concentrations or presence of solvents or other substances can trigger changes on the tertiary structure of the enzyme promoting unfolding (denaturalisation) which can be reversible or irreversible and can produce the loss of catalytic activity. Enzyme activity is not necessary related to stability, since their optimum conditions can be different [5], [6].

Additionally, some enzymes need another chemical component (non-protein) to catalyse reactions: the cofactor. It can be divided into two types: inorganic, such as Fe^{2+}, or organic/organometallic, called coenzyme, such as FAD^{+}, $NAD(P)^{+}$ or vitamin C. Coenzymes can be covalently (prosthetic group) or non-covalently (co-substrate) bound to the enzyme. They act as transient carriers, moving small components such as electrons or bigger components, like functional groups. Once the enzyme is bound to the cofactor, it is ready to transform the substrate. Some cofactors are consumed during the reaction and normally they need a regeneration system to recycle them because of their high price [1], [6].

1.1.1 Classification of enzymes

Enzymes are classified, according to the *Enzyme Catalogue*, with a four-number code (Enzyme Commission number). They are divided into six big groups (first number) depending on the type of chemical reaction that they catalyse (**Table 1.1**). The second number defines the functional group acted on. The third number corresponds to the chemical groups involved in the reaction and the last number corresponds to each enzyme that is in these sub-sub-groups [7], [8].

1.1.1.1 Oxidoreductases

Among the six different classes of enzymes, oxidoreductases are the largest class [9]. They catalyse oxidation/reduction reactions through the transfer of electrons, hydrogen or oxygen atoms [6]. Redox enzymes represent about 25 % of all known enzymes and its importance is due to their involvement in many significant pathways of the cell that need electron transfers such as photosynthesis or respiration [10], [11]. The wide range of reactions that oxidoreduc-

Table 1.1: Classification of enzymes.

Enzyme class	Type of reaction	Reaction scheme	Important subclasses
Oxidoreductases	Transfer of electrons Reduction-oxidation	$A_{red} + B_{ox} \rightleftarrows A_{ox} + B_{red}$	Dehydrogenases Oxidases Peroxidases Reductases Monooxygenases Dioxygenases
Transferases	Transfer of a chemical group	$A\text{-}B + C \rightleftarrows A + B\text{-}C$	Transferases Glycosyltransferases Aminotransferases Phosphotransferases
Hydrolases	Hydrolysis	$A\text{-}B + H_2O \rightleftarrows A\text{-}H + B\text{-}OH$	Esterases Glycosidases Peptidases Amidases
Lyases	Removal of a chemical group leaving a double bond (Non-hydrolytic)	$A + B \rightleftarrows A\text{-}B$	C-C-Lyases C-O-Lyases C-N-Lyases C-S-Lyases
Isomerases	Transfer of groups in a molecule to form isomers	$A \rightleftarrows \text{Iso-}A$	Epimerases *cis trans* Isomerases Intramolecular transferases
Ligases	Covalent linkage of two or more molecules using ATP or analogous	$A + B + XTP \rightleftarrows A\text{-}B + XDP$ X= A,G,U,C	C-C-Ligases C-O-Ligases C-N-Ligases C-S-Ligases

tases catalyse, their high specificity and efficiency synthesising optically active compounds and chiral building blocks can be of high importance for the synthesis of fine chemicals, pharmaceuticals and other value-added products [12].

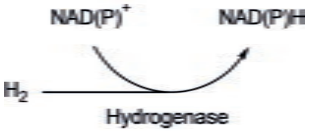
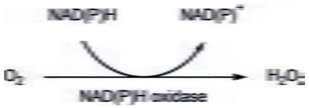
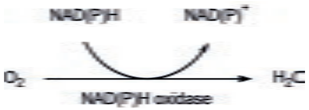
However, oxidoreductases often require stoichiometric amounts of very expensive coenzymes such as NAD(P)$^+$/NAD(P)H which make the use of efficient coenzyme recycling systems indispensable in the development of economically sustainable biosynthesis. This recycling process can be performed microbiologically, enzymatically, chemically, electrochemically and photochemically [3], [13]. Enzymatic regeneration provides benefits such as selectivity, efficiency and feasibility to couple more than one target reaction such as the co-product recycling [14] or the use of enzymatic cascades, to perform a multistep target reaction [15], [16]. Enzymatic regeneration can be achieved in two different ways: a second substrate, usually called a sacrificial substrate, can be added to be catalysed reversibly or preferably irreversibly by i) the same enzyme [17] or ii) by another biocatalyst [18]. The selection of the sacrificial substrate can lead to a coupled reaction producing a by-product or another valuated compound. Nevertheless, generally it is difficult to perform thermodynamically-favourable oxidation and reduction reactions under the same reaction conditions. Therefore, typically, a second enzyme is added to perform a coupled reaction producing a by-product, just for regenerating the coenzyme [3]. There are different typical thermodynamic-propitious enzymatic systems to regenerate NAD(P)$^+$ and NAD(P)H, some examples are showed in **Table 1.2** [19]. Another interesting method to manage a reaction which requires cofactor regeneration is by fusing the involved enzymes which may improve the regeneration of the cofactor rapidly due to its near localisation [20], [21].

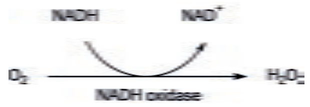
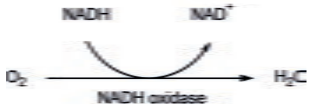
Another limitation of many oxidoreductases is that they use oxygen as a substrate, which limits the use of flammable solvents. Moreover, it requires specific reactor configurations to allow high oxygen transfer. The oxygen requirement produces another limitation related to the gas-liquid interphase since usually it can trigger enzyme unfolding and inactivation [33]. Even so, the use of oxygen is a better environmentally friendly alternative to chemical oxidants [34].

Furthermore, the main drawback that hampers the application of the oxidoreductases in many industrial processes is their low operational stability, resulting in low total turnover numbers [35], [36]. The high importance of obtaining robust enzymes is noticeable by the amount of publications aiming to improve the enzymatic stability by multiple strategies [37]–[39].

Table 1.2: Examples of NAD(P)$^+$ and NAD(P)H-dependent oxidoreductases for cofactor regeneration [19].

Enzyme	microorganism	Cofactor regenerated	Comments	K_{cat} (s^{-1}) or Vmax (U mg^{-1})	K_M for the cofactor	K_M for S	Reaction scheme	Ref.
R-Alcohol dehydrogenase	*Lactobacillus brevis*	NADPH & NADH	Commercially available. widely used for enzymatic generation of pharmaceuticals. Wild-type is NADP$^+$-dependent, mutant strongly prefers NAD$^+$.	100 s^{-1}	*	*		[22]
Formate dehydrogenase	Various native and mutants are reported. FDH of *Paracoccus sp.* 12-A native	NAD(P)H	Inexpensive sacrificial substrate. Thermostable. Favourable thermodynamically. Prefers NAD$^+$. Moderate stability, and low specific activity. Widely used industrially.	8.5 s^{-1}	36 µM (NAD$^+$)	5 mM		[23]
Phosphite dehydrogenase	*Pseudomonas stutzeri* WM88	NAD(P)H	Inexpensive sacrificial substrate. Phosphate does not inhibit PtxD. Enzyme displays a broad pH-rate maximum. prefers NAD$^+$. Favourable thermodynamically.	7.3 s^{-1}	50 µM	50 µM		[24]
Glucose dehydrogenase	*Bacillus subtilis*	NAD(P)H	Commercially available, Inexpensive sacrificial substrate. Accepts NADP$^+$ as well as NAD$^+$. Product may complicate workup procedure.	*	0.23 mM for NAD$^+$ 0.26 mM for NADP$^+$	43 mM		[25]

Enzyme	microorganism	Cofactor regenerated	Comments	K_{cat} (s^{-1}) or Vmax (U mg^{-1})	K_M for the cofactor	K_M for S	Reaction scheme	Ref.
Glucose -6-phosphate dehydrogenase	*Bacillus stearothermophilus*	NAD(P)H	Commercially available Expensive sacrificial substrate. Thermostable. Product may complicate workup procedure. Accepts $NADP^+$ and NAD^+.	*	*	280 μM		[26]
Hydrogenase	*Ralstonia eutropha* *Pyrococcus furiosus*	NADH and NADPH respectively	100% atom-efficiency. cheap sacrificial substrate. favourable thermodynamically. no carbon-based by-products produced. Technically challenging to use H_2.	*	560 μM for NAD^+ (*R. eutropha*)	11-37 μM for H2 (*R. eutropha*)		[27]
Pyridine nucleotide transhydrogenase	*Pseudomonas fluorescens*	NAD^+ and NADPH or NADH and $NADP^+$	Requires coupled reactions for both products	*	*	*	$NAD^+ + NADPH \underset{STH}{\rightleftharpoons} NADH + NADP^+$	[28]
NAD(P)H oxidase H_2O_2-forming	*Lactobacillus sanfranciscensis*	$NAD(P)^+$	Inexpensive sacrificial substrate. By-product can inactivate enzymes and/or react with others substrates or products. A catalase should be added to eliminate H_2O_2. Technically challenging to use O_2.	11 U mg^{-1}	6.7 μM	*		[29]
NAD(P)H oxidase H_2O-forming	*Lactobacillus plantarum* mutant G178R/L179R	$NAD(P)^+$	Inexpensive sacrificial substrate. Innocuous by-product. High activity and stability. Technically challenging to use O_2.	146.4 s^{-1}	9.8 μM	*		[30]

Enzyme	microorganism	Cofactor regenerated	Comments	K_{cat} (s^{-1}) or Vmax (U mg^{-1})	K_M for the cofactor	K_M for S	Reaction scheme	Ref.
NADH oxidase H_2O_2 forming	*Thermus thermophilus* HB27 (NoxV)	NAD^+	Inexpensive sacrificial substrate. By-product can inactivate enzymes and/or react with others substrates or products. A catalase should be added to eliminate H_2O_2. Technically challenging to use O_2.	15.6 s^{-1}	2.1 mM for NADH	*		[31]
NADH oxidase H_2O forming	*Streptococcus mutans* (93R and 94H)	NAD^+	Inexpensive sacrificial substrate. Innocuous by-product. High activity. Technically challenging to use O_2.	20 s^{-1} for NADPH and 25 s^{-1} for NADH	6 µM for NADPH and 9 µM for NADH	*		[32]

*Not reported.

1.1.2 Enzymatic stability

Evolutionally, enzymes have been adapted to act in their natural role, where there is a determined aqueous media with low concentrations of substrates and other products. The loss of enzymatic stability is on account of different processes that produce modifications on the structure of the enzymes, which can be boosted by the reaction conditions. These processes can produce aggregation, chemical modifications or subunit dissociation triggering the irreversible inactivation of the enzyme [35], [40].

Since oxidoreductases, specifically multimeric ones, are known to have lower robustness than many other enzymes, it is necessary to apply different technics to improve their operational stability for industrial application [35]. The main methods that can be used to achieve stable enzymes are chemical modification, protein engineering, reaction and reactor engineering and immobilisation [6], [41]. Chemical modification of enzymes can be performed through random modification of their amino acids residues through chemical mutagenesis at the DNA level or specific regions involved in the stability or activity of the enzyme. These modifications, which cannot be achieved by mutagenic techniques, have been proven to be able to improve the activity and stability towards organic solvents, extreme temperature and pH [42]. Protein engineering may also improve enzyme stability, activity or create novel functionalities, by inducing changes in the amino acidic chain of the enzyme. These modifications can be performed by directed evolution, by rational design or by the combination of both methodologies (semirational design).

Directed evolution uses random mutation methods which produces changes in the amino acid sequence of the protein (i.e. error-prone PCR) that can be beneficial or not, so it requires large screening. These methods are decaying in interest, and currently rarely used, since the random mutations make it necessary to perform repeated rounds of mutagenesis and screening that are lengthy. Rational design, which allows the modification of specific protein regions, arose with the availability of advanced computational engineering tools and the growing availability of protein structures, biochemical and molecular modelling data. These methods are used to predict mutations of the regions implied that might have an important role in the target properties of the enzyme. Then, the mutations can be performed by site-directed mutagenesis or by saturation mutagenesis at individual or multiple sites with high throughput screening, reducing considerably the screening time compared to the direct evolution [43]–[47].

Reaction engineering allows the selection of the best operational conditions where there was a compromise between the best conditions for the enzyme and for the target product formation. Reactor configuration allows the control of the reaction, maintaining the best conditions over the time [6]. Immobilisation of enzymes is a key technique, not only to improve enzyme stability but also to make a biocatalytic process economically viable which can be accomplished through the reutilisation of the immobilised enzyme [35]. Moreover, immobilisation facilitates product purification, since the enzyme can be removed easily by filtration. This is an important point since some industries (i.e. pharma), require protein-free products since proteins can produce non-desired side effects such as allergenic issues [48]. Finally, immobilisation facilitates to perform processes in other reactor configurations than batch, i.e. continuous mode.

1.1.3 Immobilisation

Immobilisation can be defined as a technique that allows the physically confinement or localisation of the biocatalyst in an defined region of the space, preserving their catalytic activity, which facilitates the re-use of a biocatalyst and/or use in a continuous mode [6]. In addition, immobilisation has been proved to provide a more robust biocatalyst by improving the stability, activity and the tolerance to organic solvents [35], [49], [50].

There are different methods to immobilise an enzyme that can be divided into immobilisation into the use of a solid pre-existent support or carrier-free methods. When the immobilisation is performed without a solid pre-existent support it usually becomes cheaper so the enzyme molecules are immobilised to themselves with a reagent (cross-linking) or confined by different techniques (encapsulation, entrapment or use of ultrafiltration membranes).

However, carrier-free techniques usually present some important drawbacks if we want to use them industrially, like poor mechanical properties (cross-linking and encapsulation), high mass transfer limitations (entrapment and cross-linking), difficult recovery of the derivatives compared to enzymes immobilised onto solid supports (cross-linking, encapsulation) or non-improvement of stability (containment by ultrafiltration membranes) [6], [51], [52] (**Figure 1.1**).

The immobilisation techniques that allow easiest reuse of enzymes in conventional reactors, are those where the enzyme is immobilised onto solid pre-existent supports, since their mechanical properties are more robust and the size of the particles is more uniform. These immobilisations can be performed by covalent or non-covalent bonds. The non-covalent interactions can be formed by weak forces like Van der Waals, stronger hydrophobic forces, or ionic bonds. These immobilisations are typically simple and rapid to carry out, retaining high activity

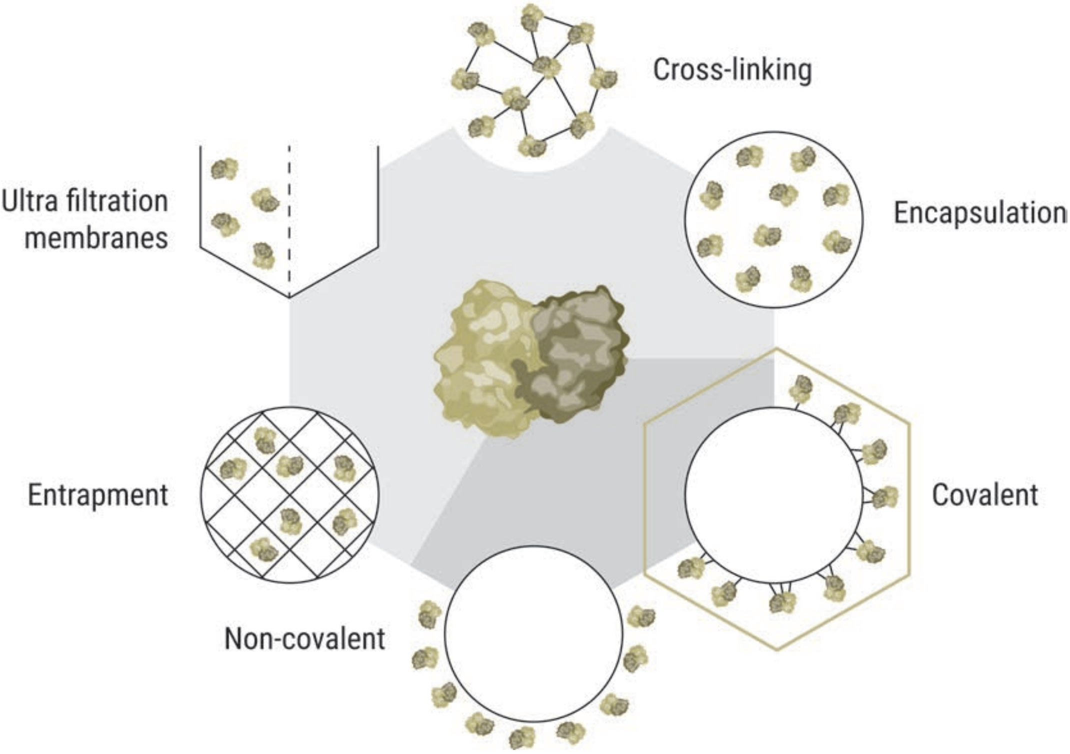


Figure 1.1: Scheme of the different immobilisation methods. Dark grey and light grey: immobilisation onto and without pre-existent support. Highlighted: the selected immobilisation method used in this thesis.

and allow removal of the enzyme once it loses its activity to reuse the carrier. However, when the enzyme is non-covalently immobilised , depending on the reaction conditions, the enzyme can be released from the support. Covalent immobilisation is performed by promoting chemical reaction between the superficial amino acids residues of the enzymes and the functional groups of the support. This immobilisation method may produce multipoint strong bonds. If multipoint immobilisation is produced, the rigidity of the enzyme increases which usually stabilises its structure resulting in an improvement of stability. This immobilisation is interesting specially when we want to immobilise multimeric enzymes (as are most of the oxidoreductases) since the increase of rigidity slows down the mechanisms of irreversible inactivation. In the general inactivation mechanism, the native structure of the enzyme is in equilibrium with a semi-unfolded structure which suffers irreversible inactivation processes. The rigidity shifts the balance to the native structure and the lower concentration of semi-unfolded structure trigger the reduction of the irreversible inactivation rate [6], [53], [54]. However, the irreversible immobilisation presents also some drawbacks: i) if the covalent bonds interact with the active site, they may cause loss of catalytic activity, ii) as the enzyme is immobilised covalently, once the enzyme is deactivated, both, the enzyme and the support are rendered unusable. Therefore, it is necessary to use new carrier to immobilise more enzyme. This is an important drawback particularly since iii) the support often is expensive and can increase the price of the biocatalyst more than 4-fold. Therefore, this extra-cost needs to be compensated by enzyme recycling [55]–[58].

Enhanced operational stability of different covalently immobilised oxidoreductases has been demonstrated by performing successive reaction cycles (of industrial interest) in which process metrics such as the biocatalyst yield have been improved (up to 8 cycles and more than 3-fold improvement) compared to the use of soluble enzyme [59], [60].

Moreover, when more than one enzyme is required, (i.e. multi-enzymatic reactions, enzyme-coupled reactions for cofactor regeneration), a good immobilisation strategy is the co immobilisation of the enzymes onto the same support. Co-immobilisation presents some advantages, in contrast with individual immobilisation, such as enhanced reaction kinetics by optimising the catalytic turnover due to the physical proximity between the enzymes. However, co-immobilisation is not always viable because each enzyme has specific optimal ranges of activity and stability which can be incompatible. Moreover, these conditions might not be compatible with some immobilisation methods and/or supports. Therefore, screening of conditions and immobilisation methods is often needed. Moreover, co-immobilisation should preserve the catalytic activity and improve the operational stability of each enzyme. Obtaining good immobilised derivatives preserving the activity and improving the stability of each enzyme is not always possible so co-immobilisation only will be considered when this could be possible [61].

Some examples of successful reusability of co-immobilised oxidoreductases are: the use of co-immobilised glycerol dehydrogenase and NADH oxidase which gave a higher utilisation efficiency of the cofactor and higher operational stability compared to the enzymes immobilised separately [62]. A cytochrome p450 co-immobilised with a cofactor regenerator (GDH) onto different supports, which led to more than a 2.3 fold biocatalyst yield improvement compared to the soluble forms, improving the operational stability when 5 reaction cycles were performed [63]. Another example is the co-immobilisation of 3 enzymes (galactose oxidase, catalase and horseradish peroxidase) where 30 reaction cycles could be performed in the synthesis of 2,5-Diformylfuran, a building block for many industries [64]. Another interesting example is the simultaneous production of two value-added products (gluconic acid and xylonic acid) from glucose and xylose by co-immobilised glucose dehydrogenase and xylose dehydrogenase

Table 1.3: Some examples of successful reusability of co-immobilised oxidoreductases.

Co-immobilised enzymes	Product/s	Improvement	Ref.
Glycerol dehydrogenase + NADH oxidase	1,3-dihydroxyacetone (DHA)	10 reaction cycles. Conversion improved 2.5-fold compared to soluble form	[62]
Cytochrome p450 + Glucose dehydrogenase	10, 11 and 12-hydroxy-laureate	5 reaction cycles. 2.3-fold biocatalyst yield	[63]
Galactose oxidase + catalase + horseradish peroxidase	2,5-Diformylfuran	30 reaction cycles.	[64]
Glucose dehydrogenase and xylose dehydrogenase	gluconic acid and xylonic acid	10 reaction cycles.	[65]

which improved the stability and could be recycled up to 10 reaction cycles [65] (**Table 1.3**).

The benefits of co-immobilisation are also present in the immobilisation of fused enzymes due to the close local proximity of the enzymes. An example of this type of immobilisation is the immobilisation of cyclohexanone monooxygenase fused with phosphite dehydrogenase (cofactor regenerator) where up to 18 reaction cycles and 17-fold improvement of biocatalyst yield could be obtained [16].

1.1.4 Industrial application of enzymes

Enzyme stability improvement through all the previously mentioned methods may enable the employment of these proteins in industrial processes. Although natural enzymatic processes are present in industry since ancient times, industry still needs robust and stable enzymes to make bioprocesses economically viable. Over the past decades, the improvements in enzyme research promoted by the advances in technologies for enzyme discovery, genetic tools, analytical technologies, and machine learning algorithms, has enabled to develop enzymes where no one would have expected just decades ago [66]–[70]. The constantly growing of new developed enzymes has triggered the progressive implementation of biocatalytic processes in various sectors of industry such as food, pharmaceuticals, energy, textiles, leather, paper and detergent. The industrial interest is related to the catalytic advantages of the enzymes such as the high efficiency (k_{cat}/K_M) the mild temperature and pressure conditions required and the high product selectivity which is a very important factor for the synthesis of chiral compounds with high enantioselectivity. These characteristics translate to a reduction in process time, reduction of waste generation, increase in safety and a reduction of energy consumption, all of which reduce considerably the process costs. Moreover, the growing public concern about the environment also influences industry to implement greener and eco-friendly processes. Additionally, the use of enzymatic processes may produce natural labelled products, which are increasingly sought by consumers who are willing to pay a premium for products produced by natural means [43], [58], [71], [72].

Enzymes have been present in multiple industrial processes for decades, especially hydrolases which are the mayor class of enzymes present on the market. Its success is basically due to four reasons: i) their high potential in different processes which can be applied in different industries, ii) their inexpensive price, iii) their high stability and iv) their non-cofactor dependency [6], [73].

The presence and/or potential of oxidoreductases in industrial processes is distributed in various applications: i) in the biodegradation/bioremediation to degrade contaminant compounds, ii) the synthesis of polymers, iii) in the construction of biosensors, iv) in the oxidation/ reduction of organic substrates to obtain high added value products and v) in the regeneration of cofactors, which is typically coupled with the target reaction [13].

Enzymatic bioremediation is focused in the use of oxidoreductases (such as peroxidases and laccases) in pollutant degradation due to their high efficiency compared to other methods [74]. The biosynthesis of polymers such as polyphenols, polyanilines or vinyl polymers can be performed by oxidoreductases such as peroxidases, laccases, tyrosinases or glucose oxidases [75].

Oxidases are the main oxidoreductases used in the construction of biosensors. They are widely used since they do not require cofactor recycling. Oxidases use molecular oxygen as electron acceptor producing hydrogen peroxide. Both, dissolved oxygen and hydrogen peroxide, are easily and rapidly measurable by amperometric-based biosensors. Therefore, oxidases such as glucose oxidase, lactate oxidase, alcohol oxidase are widely used to measure glucose, lactate and alcohol [76], [77].

Less utilised are, other oxidoreductases, such as reductases (nitrate reductase to analyse nitrates in food) [78], dehydrogenases (for example, alcohol dehydrogenase to measure ethanol, which is interesting in many industries) [79], or peroxidases (such as horseradish peroxidase, to detect a mycotoxin that can be present in food and is carcinogenic for humans) [80], are also used in biosensors.

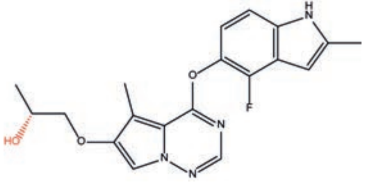
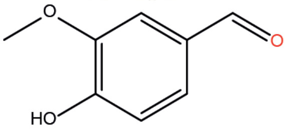
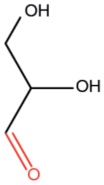
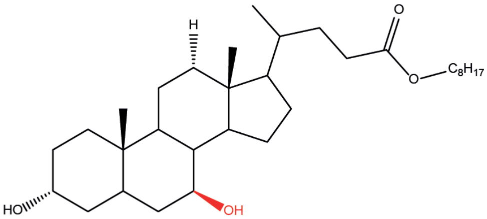
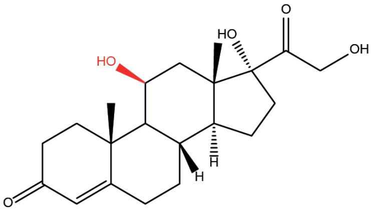
The oxidation/reduction of organic compounds, such as alcohols to aldehydes or ketones, is also an important target to replace chemical processes which generate large amounts of metal waste through expensive oxidizing agents and other non-eco-friendly reagents and extreme conditions [81]. In order to demonstrate the importance and the presence of oxidoreductases in the industrial biosynthesis of high value-added organic compounds, some examples of enzymatic reactions patented are shown in **Table 1.4**. The examples show ketoreductases or dehydrogenases which can produce high yields (even higher than through the chemical processes available) and/or excellent purity of chiral precursors interesting for pharma industry [82], [83]. For example, an engineered isoeugenol monooxygenase could catalyse the transformation of isoeugenol to vanillin and acetaldehyde at concentrations up to 20 g L^{-1} [84]. Another example is the conversion of glycerol, that represents the major by-product of biodiesel production, by an alcohol oxidase mutant to glyceraldehyde. This compound could be further transformed through an aldolase to glyceric acid, an important building block for the fine chemical and pharma industries [85].

Nevertheless, the presence of enzymes, especially of oxidoreductases, in industry is still low in spite of their high potential, catalysing highly selective reductions and oxidations. As mentioned above, the main drawbacks of oxidoreductases that must be solved for its industrial implementation are the low stability and the use of highly expensive cofactors which need to be regenerated. This is a gap that researchers are actually focused on, applying available technologies in order to solve it [6], [58], [73].

1.2 ROBOX project

This thesis was framed in the European ROBOX project (ref. 635734) entitled "Expanding the industrial use of Robust Oxidative Biocatalysts for the conversion and production of alcohols" where a consortium of scientists and industries worked together aiming to bring closer the use of oxidoreductases to industrial implementation. The objective of the ROBOX project was to demonstrate the economic viability of bio-transformations of four types of

Table 1.4: Examples of patented enzymatic reactions with engineered modified oxidoreductases. In red the modified group.

Enzyme type	Obtained product	Comments	Ref.
Ketoreductase		Chiral alcohol precursor for pharmaceuticals useful as inhibitors of vascular endothelial growth factor receptor-2, useful as anti-cancer agent.	[82]
Isoeugenol monooxigenase (as whole cells)		The second most important flavour in the world, useful in food and pharma industries.	[84]
Alcohol oxidase		The product could be converted with an aldolase to glyceric acid which is an important building block for fine chemicals and pharmaceuticals.	[85]
7β-hydroxysteroid dehydrogenase		The enzyme could be applied in a redox-neutral biocascade for the synthesis of Ursodeoxycholic acid which solubilizes cholesterol gallstones and improve liver function in cholestatic diseases.	[83]
Steroid 11β-hydroxylase (monooxygenase)		Monooxygenases are used to hydroxylate steroids such as hydrocortisone which is used as a treatment of adrenocortical insufficiency, rheumatoid arthritis, asthma, etc.	[86]

oxidoreductases: P450 monooxygenases (P450s), Baeyer-Villiger monooxygenases (BVMOs), alcohol dehydrogenases (ADH) and alcohol oxidases (AOX). The target reactions were focused on pharma, fine & specialty chemicals and materials applications. The specific objectives of the ROBOX project were divided into five different work packages (WP) (Figure 1.2).

The first work package was focused on the identification and engineering of new robust oxidoreductases aiming to improve their stability, activity and efficiency through protein engi-

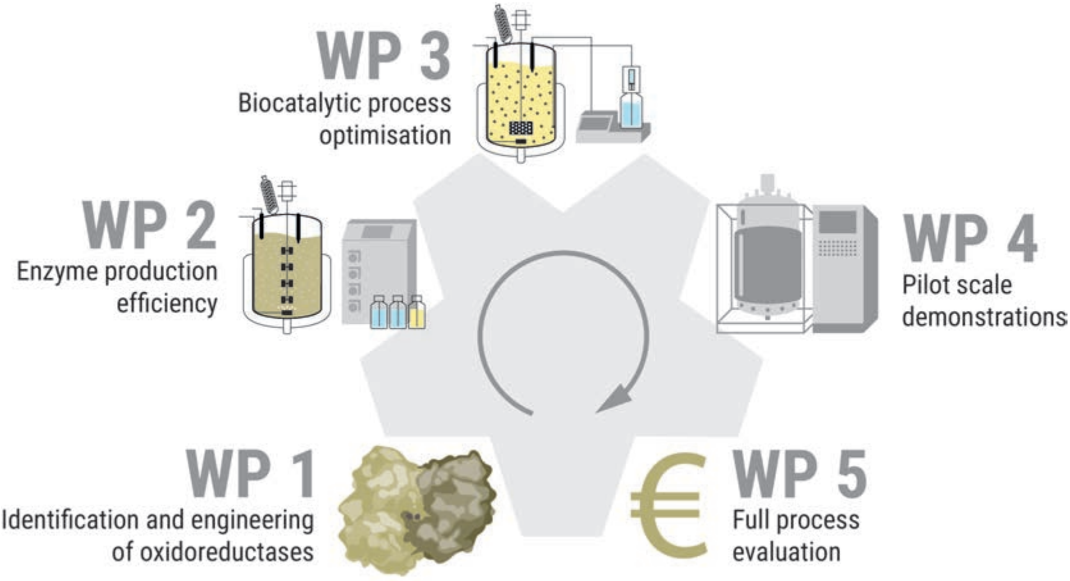

WP 3
Biocatalytic process optimisation
WP 2
Enzyme production efficiency
WP 4
Pilot scale demonstrations
WP 1
Identification and engineering of oxidoreductases
WP 5
Full process evaluation
€

Figure 1.2: Scheme of the different work packages of ROBOX project.

neering techniques including i.e. computationally assisted engineering of enzymes. WP2 established competitive fermentative platforms for efficient high-level production of the selected enzymes of WP1 through advanced fermentation production technologies with recombinant hosts. Then, the obtained enzymes in WP2 were used by WP3 to develop efficient biocatalytic processes via reaction and reactor optimisation. Moreover, WP3 immobilised the selected enzymes, with different methods, in order to improve their stability and to allow biocatalyst re-use. Cofactor regeneration was also studied in WP3. WP4 was focused on scaling-up the best candidates of WP3 in order to demonstrate the techno-economic viability of the biocatalytic oxidations for market development and exploitation. Finally, WP5, evaluated the full process (costs, environmental sustainability, energetic performances, comparison with conventional processes, etc.) of the demonstrated biocatalytic processes and technologies of the ROBOX project in order to bring closer them to different markets (pharma, nutrition, fine/speciality chemicals and materials).

The target reactions proposed by the ROBOX project, its applications and enzymes involved are depicted in Figure 1.3. The target reactions of this thesis are highlighted.

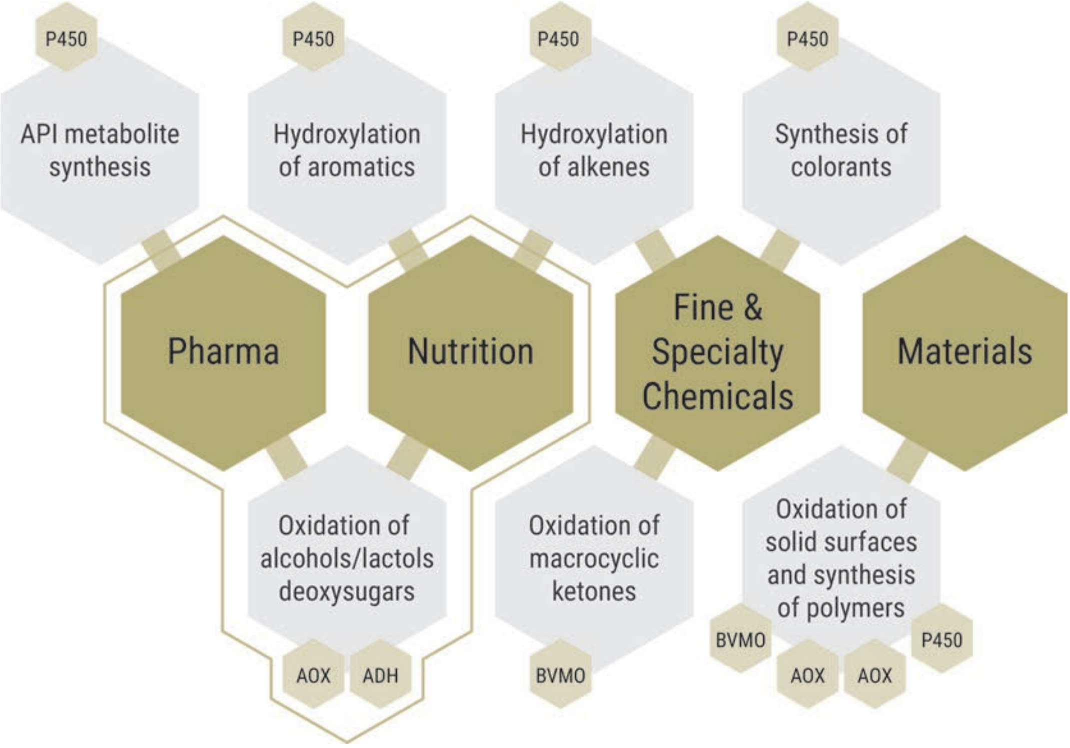

P450
API metabolite synthesis
P450
Hydroxylation of aromatics
P450
Hydroxylation of alkenes
P450
Synthesis of colorants
Pharma
Nutrition
Fine & Specialty Chemicals
Materials
Oxidation of alcohols/lactols deoxysugars
AOX
ADH
Oxidation of macrocyclic ketones
BVMO
Oxidation of solid surfaces and synthesis of polymers
BVMO
P450
AOX
AOX

Figure 1.3: Scheme of the target reactions of the ROBOX project with the enzymes involved and their application to the market. Highlighted: reactions, enzymes and applications of this thesis.

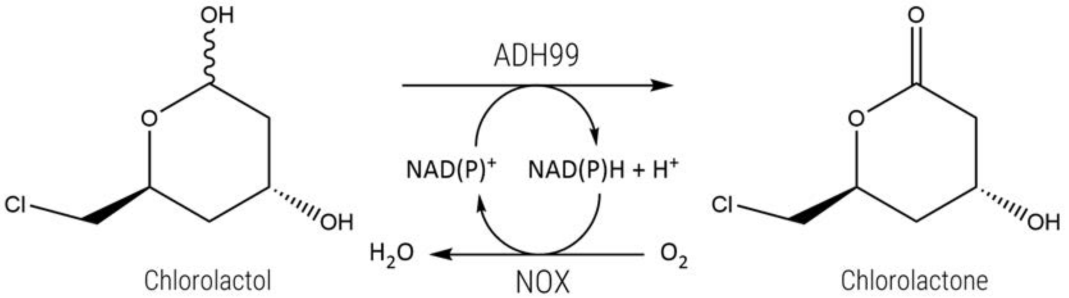
1.3 Target reactions of this thesis

The ROBOX project was focused on 11 target reactions catalysed by the 4 oxidoreductases mentioned above. This thesis was centred on 2 of these reactions within WP3:

- Synthesis of chlorolactone, a statin precursor, by an ADH-catalysed oxidation of chlorolactol.
- Synthesis of vanillin, a flavour compound, by an AOX-catalysed oxidation of vanillyl alcohol.

1.3.1.1 Chlorolactol oxidation to Chlorolactone by an Alcohol dehydrogenase

Chlorolactone (chloromethyl)tetrahydro-2H-pyran-2,4-diol) is an important chiral building block in the synthesis of statins, cholesterol reducing drugs [87], [88]. Its enzymatic synthesis from chlorolactol ((4*R*,6*S*)-6-(chloromethyl)tetrahydro-2H-pyran-2,4-diol) is interesting because this substrate can be synthesised from scratch enzymatically being a cost effective, environmentally friendlier and more sustainable compared to chemical synthesis [89], [90]. This compound can be obtained by oxidation of chlorolactol catalysed by an alcohol dehydrogenase. $NAD(P)^+$ is required in this enzymatic strategy which can be regenerated by coupling with an NADPH oxidase. In the present thesis, the synthesis of chlorolactone from chlorolactol has been studied using an $NADP^+$-depending ADH (ADH99) and a NADPH oxidase (NOX) (**Scheme 1.1**). InnoSyn was the ROBOX end-user of this target reaction.



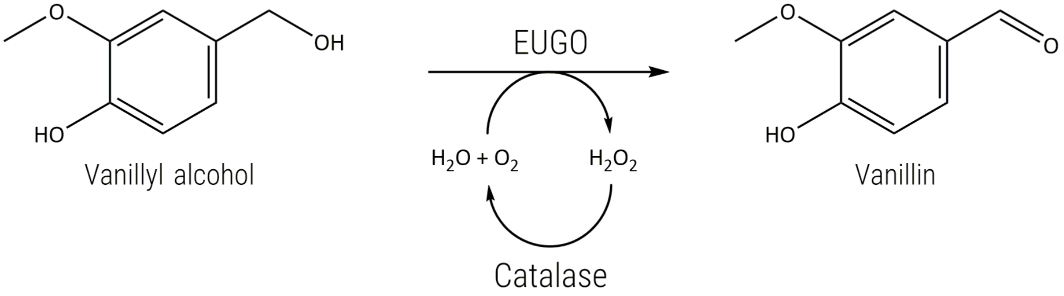
Scheme 1.1: Chlorolactone synthesis though chlorolactol by ADH99 and NOX for cofactor regeneration.

Recombinant alcohol dehydrogenase 99 (ADH99) is a $NADP^+$-dependent dehydrogenase (EC 1.1.1.1) produced in *E. coli* by C-LEcta. It is constituted by four homomonomers of 25 kDa each with an isoelectric point (pI) of 6.08 (**Table 1.5**).

The applied NAD(P)H oxidase (NOX) is a recombinant oxidase from *Streptococcus mutants* (EC 1.6.3.2) produced in *E. coli* by DSM/InnoSyn [32]. NOX catalyses the oxidation of NAD(P)H using O_2 as an electron acceptor, producing H_2O. It is a monomer of 50 kDa with 2 points of mutation that contains the FAD cofactor as a prosthetic group. This enzyme has a pI of 5.4 (**Table 1.5**).

1.3.1.2 Vanillin synthesis through vanillyl alcohol oxidation by Eugenol oxidase

Vanillin which is the component of vanilla that produces its organoleptic properties is obtained naturally by agricultural production [91]. However, difficulties associated with its farming, such as the need to hand pollinate, and its high demand, triggered the necessity to produce it by other methods [92]–[94]. One of them is the enzymatic oxidation of vanillyl alcohol to vanillin by an oxidase. This enzymatic strategy for vanillin synthesis has been studied in the present thesis using an eugenol oxidase (EUGO). This enzymatic oxidation produces hydrogen peroxide as a by-product which can be eliminated by coupling with a catalase (**Scheme 1.2**). InnoSyn and Givaudan were the ROBOX end-users of this target reaction.

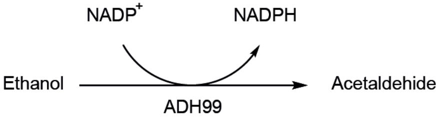
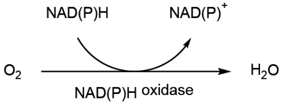
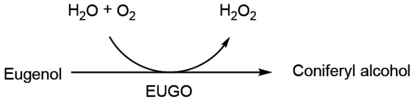
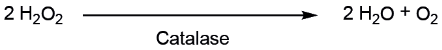


Scheme1.2: vanillyl alcohol oxidation to vanillin catalysed by EUGO. Hydrogen peroxide is degraded by catalase.

The applied eugenol oxidase (EUGO) is a wild-type alcohol oxidase from *Rhodococcus jostii* strain RHA1 [95], [96]. It is a dimer (59 kDa) containing FAD as a prosthetic group. Its native reaction is the oxidation of eugenol to coniferyl alcohol [96]. The enzyme was cloned in *E. coli* by RUG and supplied by InnoSyn. This enzyme has a pI of 4.8 (**Table 1.5**).

The catalase applied in this work is a commercial peroxidase from bovine liver (EC 1.11.1.6) supplied by Merck. It is a tetramer of 4 equal subunits with 60 kDa each. Each subunit contains an iron bond to a protoheme IX group. Catalase catalyses the decomposition of hydrogen peroxide to water and oxygen (**Table 1.5**).

Table 1.5: Enzymes employed in this thesis.

Enzyme	microorganism	Subunits	Cofactors	Molecular weight (KDa)	pI	Supplier	Native reaction
Alcohol dehydrogenase 99 (ADH99) (engineered)	-	homotetramer	NADP$^+$	25 each one	6.08	C-LEcta	 NADP$^+$ NADPH Ethanol Acetaldehide ADH99
NAD(P)H oxidase (NOX) (engineered)	*Streptococcus mutans*	Monomer	NADP(H) FAD (Prosthetic group)	50	5.4	DSM/ InnoSyn	 NAD(P)H NAD(P)$^+$ O_2 H_2O NAD(P)H oxidase
Eugenol oxidase (EUGO) (wild type)	*Rhodococcus jostii* strain RHA1	Dimer	FAD (Prosthetic group)	58.7	4.8	InnoSyn	 $H_2O + O_2$ H_2O_2 Eugenol Coniferyl alcohol EUGO
Catalase	bovine liver	homotetramer	Each subunit contains an iron bond to a protoheme IX group	60 each one		Merck	 $2 H_2O_2$ $2 H_2O + O_2$ Catalase

A
ADH-E
NOX-G 300
ADH-M 300
NOX-M 300
NOX-G 200
ADH-M 200
NOX-G 75
ADH-M 75
NOX-G 50
ADH-M 50
100
Soluble

OBJECTIVES

2

The main objective of the present work is to bring the implementation of two target oxidoreductase catalysed biocatalytic processes closer to industrial scale, aiming to develop greener alternative processes. This thesis is focused on improving the process metrics of these two reactions of industrial interest, via immobilisation and reaction engineering.

The specific objectives are:

Chlorolactone synthesis catalysed by ADH99:

- To characterise and immobilise an alcohol dehydrogenase (ADH99) and the co-factor regenerator, an NADPH oxidase (NOX), onto different supports.
- To study the stability of the immobilised derivatives under reaction conditions.
- To characterise the maximum loading of both enzymes onto the selected supports.
- To select the best immobilised derivative in terms of stability and enzyme capacity.
- To optimise the conditions of the target reaction, the oxidation of a chlorolactol to chlorolactone.
- To study the reusability of the immobilised enzyme derivatives.

Vanillin synthesis catalysed by EUGO:

- To characterise and immobilise an eugenol oxidase (EUGO) onto different supports.
- To study the stability of the immobilised enzyme derivatives under reaction conditions.
- To characterise the maximum loading of enzyme onto the selected supports.
- To study the reusability of the immobilised enzyme derivative in the oxidation of vanillyl alcohol to vanillin.
- To compare the reusability of the immobilised enzyme derivative with alternative reaction conditions provided by the end-user InnoSyn.

MATERIALS AND METHODS

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3.1 Materials and immobilisation supports

β-Nicotinamide-adenine dinucleotide phosphate (β NADPH) was purchased from Bontac Bio-engineering (Shenzhen) Co., Ltd. Antifoam glanapon 2000 was supplied by Busetti and Co GmbH (Wien, Austria). All other reagents were supplied by Merck.

Amino functionalised agarose (amino-agarose) and non-functionalised agarose 4BCL (spherical beads with diameter of 50-150 μm), used to obtain epoxy-agarose-UAB M1 and M2, were obtained from Agarose Beads Technology® (ABT®) brands.

Purolite® supports, methacrylate matrix activated with amino (ECR8409 and ECR8415, with a pore size of 600-1200 Å and 1200-1800 Å, respectively) and epoxy groups (Praesto epoxy 45), were generously donated by Purolite® Life Sciences (Bala Cynwyd, PA, USA).

Eupergit® CM, methacrylate matrix activated with epoxy groups, was supplied by Merck.

3.2 Enzymes

Alcohol dehydrogenase-99 (ADH99) and NOX variant from *Streptococcus mutans* were provided as a lyophilised and cell free extract, by c-LEcta and DSM/InnoSyn, respectively.

Eugenol oxidase from *Rhodococcus jostii* (EUGO) was provided by InnoSyn B.V. (The Netherlands) as *Escherichia coli* lysates.

Commercial catalase from bovine liver was supplied by Merck.

3.3 Methods

3.3.1 Protein and enzyme content

Total protein content of ADH99, NOX and EUGO were analysed by the Bradford method using bovine serum albumin as standard [97]. Lyophilised ADH99 was dissolved in 50 mM potassium phosphate, pH 7.0 and centrifuged at 13400 rpm for 1 min. The supernatant was used to determine the total protein. NOX and EUGO were used without further treatment.

Enzyme content was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (NuPage 12 %, Invitrogen, USA) run in a Mini-PROTEAN II apparatus (BioRad, USA) following the protocol of Laemmli *et al.* [98]. Low range protein markers were used for molecular weight determination. Gels were stained using Comassie G250 colloidal stain solution [34 % (v v^{-1}) ethanol, 2 % (v v^{-1}) H_3PO_4, 17 % (w v^{-1}) NH_4SO_4 and 0.066 % Comassie G250] and the Image LABTM software (BioRad, USA) was used for image processing.

3.3.2 Enzymatic activity assays

The activity of ADH99 was measured spectrophotometrically at 340 nm following the consumption of NADPH (ε = 6.22 mM^{-1} cm^{-1}) during the reduction of ethylacetoacetate. A sample of enzyme preparation (50-150 μL) was added to a cuvette with 800-900 μL of substrate and diluted to a final concentration of 100 mM ethylacetoacetate dissolved in 500 mM potassium phosphate, pH 7.0 and 50 μL of NADPH dissolved in distilled water (final concentration of 0.3 mM), at 30 ºC. One unit of ADH99 activity (U) is defined as the amount of enzyme required to convert 1 μmol of NADPH per minute at the conditions described above.

NOX activity was measured spectrophotometrically at 340 nm following the consumption of the substrate, NADPH (ε = 6.22 mM^{-1} cm^{-1}). A sample of enzyme preparation (50 μL) was diluted with 900 μL of potassium phosphate buffer to a final concentration of 500 mM, pH 6.0 and 50 μL of NADPH dissolved in distilled water (final concentration of 0.35 mM), at 30 ºC. One unit of NOX activity (U) is defined as the amount of enzyme required to convert 1 μmol of NADPH per minute at the conditions described above.

The activity of EUGO was measured spectrophotometrically at 340 nm following the production of vanillin (ε = 27 mM^{-1} cm^{-1}) from vanillyl alcohol. A 50 μL aliquot of enzyme sample was added to a cuvette with 950 μL of vanillyl alcohol dissolved to a final concentration of 0.5 mM in 50 mM glycine-NaOH, pH 9.5 at 30 ºC. One unit of EUGO activity (U) is defined as the amount of enzyme required to produce 1 μmol of vanillin per minute at the conditions described above.

Catalase activity was measured spectrophotometrically at 240 nm following the consumption of the substrate, H_2O_2 ($\varepsilon = 0.0383\ mM^{-1}\ cm^{-1}$). A 50 μL aliquot of enzyme sample was combined with 950 μL of 20 mM H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0 at 25 ºC. One unit of catalase activity (U) is defined as the enzyme required to convert 1 μmol of H_2O_2 per minute at the conditions defined previously.

Activity assays were carried out using a Cary 50 Bio UV-visible spectrophotometer (Palo Alto, USA).

Activity assays were carried out using 1.5 mL cuvettes suitable for UV analysis. When the activity of ADH99, NOX and EUGO immobilised derivatives was measured, double of each volume was added and 3 mL cuvettes were used with magnetic stirring to maintain a proper suspension of the derivatives during the measurement.

3.3.3 Activity and stability of the enzymes under different pH conditions

The effect of pH on ADH99, NOX and EUGO activity was determined by measuring their enzymatic activity, as described above, under different reaction pH conditions (pH 5.0 to 9.0 for ADH99 and NOX and pH 5.0 to 10.0 for EUGO). In the case of EUGO, the molar extinction coefficient was determined (Appendix **Table 8.1**) at each pH in order to analyse the activity accurately. All reagents were dissolved in buffers of different pH, except NADPH and the enzymatic preparations which were dissolved in distilled water. The 100 mM buffers used were sodium acetate for pH 5.0 and 5.5, potassium phosphate for pH 6.0 to 8.0, Tris-HCl for pH 9.0 (for ADH99 and NOX) and Glycine-NaOH for pH 8.5-10 (EUGO). Experiments were carried out in duplicate. Error bars correspond to standard error.

The effect of pH on enzyme stability was studied under a pH range from 5.0 to 8.5 (ADH99 and NOX) and 5.0 to 10.0 (EUGO). About 1-2 U mL^{-1} of each enzyme was added to a 100 mM buffer solution with a final volume of 10 mL. Samples were incubated on a roller (Movil-Rod Selecta S.A.) for 24 hours at 25 ºC. The buffers used were sodium acetate for pH 5.0 and 5.5, potassium phosphate for pH 6.0 to 8.0, Tris-HCl for pH 8.5 and 9.0 and sodium bicarbonate (pH 10.0). The activity of the samples was analysed, as described above, at 0, 0.5, 1, 2, 4, 8 and 24 hours. Experiments were carried out in duplicate. Error bars correspond to standard error.

3.3.4 Effect of hydrogen peroxide on EUGO stability

In order to evaluate the effect of the hydrogen peroxide (formed as a by-product in vanillyl alcohol oxidation) on EUGO stability, experiments were performed in the presence of hydrogen peroxide. 1 U EUGO mL^{-1} was incubated under reaction conditions (30 % acetone, 50 mM potassium phosphate, pH 7.5, mild agitation conditions, 25 ºC), with different concentrations of hydrogen peroxide (400, 200, 100, 50, 25, 10 and 0 mM) for 24 hours. EUGO activity was analysed, as described above, at different times. Experiments were performed in duplicate.

3.3.5 Immobilisation of enzymes

Prior to immobilisation, Eupergit® CM (dry stocked support), was hydrated, incubating the support with distilled water in a 1:10 (v v^{-1}) support-water ratio for 24 h at 25 ºC. Then, all the supports were washed three times with 20 volumes of distilled water with respect to the volume of the support, removing the liquid by filtration, and two times with the corresponding immobilisation buffer. As all the supports were used hydrated and they have high porosity, they were measured as liquids taking into account their density. Then, supports were resuspended with immobilisation buffer in a 1:10 (v v^{-1}) support-buffer ratio containing the enzymatic preparation prepared as follows: Lyophilised ADH99 was dissolved in 50 mM potassium phosphate at pH 7.0 and centrifuged at 13400 rpm for 1 min. The supernatant was used as enzymatic preparation for immobilisation experiments. NOX and EUGO were used without any treatment. Preparations were incubated on a roller (Movil-Rod Selecta S.A.) at 25 ºC for all the immobilisations. Experiments were performed in duplicate.

In order to characterise the immobilisations, they were performed with low units of activity where diffusional limitations are not present. In order to ensure the absence of diffusional limitations, immobilisations with all supports were performed at different low loadings of units (2.5-30 U mL^{-1} support). When two different loadings showed the same immobilisation yield and retained activities, it could be affirmed that there were not diffusional limitations at these loadings.

During the immobilisation process, samples of supernatant and suspension were taken at different time points to test EUGO activity during the immobilisation course. Once the enzyme was immobilised onto the support, the immobilised derivative was washed with 150 mL of immobilisation buffer and filtered with a fritted glass filter to remove the non-covalently attached enzyme and residual water, leaving moist beads.

The immobilised derivatives were washed with 150 mL of reaction buffer and used immediately.

Immobilisation yield and retained activity values were calculated using the following

$$\text{Immobilisation yield (\%)} = \frac{\text{Initial native enzyme activity - Final supernatant activity}}{\text{Initial native enzyme activity}} \text{ x } 100$$

$$\text{Retained activity (\%)} = \frac{\text{Final suspension activity - Final supernatant activity}}{\text{Initial native enzyme activity}} \text{ x } 100$$

equations and activity data determined as described above:

Final supernatant activity: activity of the supernatant when the immobilisation is finished.

Final suspension activity: activity of the suspension (supernatant and immobilised derivative) when the immobilisation is finished.

3.3.5.1 Immobilisation of ADH99, NOX and EUGO onto amino functionalised supports

Immobilisation onto amino-agarose was performed by typically mixing 9 mL of 25 mM potassium phosphate, pH 6.0 for EUGO and pH 6.5 for ADH99 and NOX, with 10 U of enzyme mL^{-1} support and 1 mL of support for 15-30 min for ionic adsorption to the support. After that, 5 mL of 200 mM (N-(3-dimethylaminopropyl)-N-ethyl) carbodiimide (CDI) was prepared in the same immobilisation buffer adjusting the pH to 6.0 or 6.5 with HCl (1 M), respectively. A sample of supernatant (0.375-5 mL, depending on the desired final concentration) was replaced by the CDI preparation to achieve the desired CDI concentration for EUGO (25 mM) and ADH99 and NOX (7.5-100 mM). The mixture was incubated under mild agitation conditions for 2 and 3 hours, respectively, at 25 ºC to covalently immobilise the enzyme. Finally, in order to desorb the non-covalently attached enzyme, 1 M NaCl was added as solid to EUGO immobilisation and it was incubated for 1 h at 25 ºC. In the case of ADH99 and NOX immobilised derivatives, they were washed and filtered 3 times with 20 mL of 1 M potassium phosphate (60 mL), pH 6.5 and 2 times with 20 mL of 25 mM potassium phosphate (40 mL), pH 6.5. The immobilised derivatives were then resuspended with immobilisation buffer 10 % ($v\ v^{-1}$) (total volume: 10 mL) and the activity of the suspension was analysed.

Immobilisation of ADH99 and NOX onto Purolite® ECR8409 and ECR8415 were performed following the same procedure using a final concentration of 10 mM of CDI.

3.3.5.2 Immobilisation of ADH99, NOX and EUGO onto epoxy functionalised supports

Immobilisation of ADH99 and NOX onto Eupergit® CM was carried out as follows: the enzyme preparation (10 U of enzyme mL^{-1} support) was mixed, typically, with 1 mL support and 9 mL of 1 M potassium phosphate of the desired pH (7.0 and 8.0) at 25 ºC. Immobilisations were performed under mild agitation conditions during different incubation times (1-21 h). Once the enzyme was immobilised, 0.2 M of β-Mercaptoethanol (140 μL) was added to the mixture and incubated for 4 h at 4 ºC in order to block epoxy groups that have not formed covalent bonds with the enzyme. The immobilised derivatives were washed 3 times with 20 mL of 25 mM potassium phosphate buffer, pH 7.0 or 8.0 and resuspended in an equal volume of 1 M potassium phosphate, pH 7.0 or 8.0.

Two different epoxy functionalised supports were used: Functionalisation method 1, containing 30 µmol of epoxy groups per g of support (F.M. 1) described by Axarli *et al.* [99] and functionalisation method 2, containing 80 µmol of epoxy groups per g of support (epoxy-agarose-UAB M2) described by Sunberg *et al.* [100].

Immobilisation of ADH99 and NOX onto epoxy-agarose-UAB M2 was performed following the same procedure at pH 8.0 during 4 hours.

The immobilisations of EUGO onto epoxy functionalised supports (epoxy-agarose-UAB M1, M2 and Praesto epoxy 45) were performed using the same protocol (pH 7.5-8.0 and 4 hours of incubation).

The immobilised derivatives were washed with the buffer needed and used immediately.

3.3.6 Stability of soluble and immobilised enzymes under reaction conditions

Stabilities of ADH99 and NOX under reaction conditions (50 mM potassium phosphate, pH 6.0, 28 ºC, mild agitation) and storage conditions (50 mM potassium phosphate, pH 6.5 for NOX and pH 8.0 for ADH99, 4 ºC) were studied by analysing the enzymatic activity over time for soluble enzymes and enzymes immobilised onto Eupergit® CM, amino-agarose and epoxy-agarose-UAB M1 (10 % v v^{-1}). Experiments were performed in duplicate.

Stability of EUGO under reaction conditions (30 % acetone in 50 mM potassium phosphate buffer pH 7.5, 25 ºC, mild agitation conditions) and storage conditions (25 mM potassium phosphate buffer pH 6.0, 4 ºC) was also studied for free enzyme and enzyme immobilised onto the selected supports (10 % v v^{-1}). Experiments were performed in duplicate.

3.3.7 Determination of maximum enzyme loading on the selected supports

The study was performed by increasing the offered units of activity, of ADH99, NOX or EUGO, to the selected supports following the corresponding immobilisation procedure until the activity measured in the supernatant after the immobilisation was higher than 10 % of initial activity offered. Experiments were carried out in duplicate.

3.3.8 Target reaction: Chlorolactol oxidation by ADH99 and NOX

3.3.8.1 Optimisation of reaction conditions to perform chlorolactol oxidation with soluble enzymes

A response surface methodology was applied to assess the effect of the substrate concentration, ADH99:NOX ratio and reaction time on different process metrics. Reactions were performed in a 10 mL tubular mini-reactor as follows: 50 mM potassium phosphate, pH 6.0 (controlled by automatic titration with 1 M NaOH), 1.3 mM NADPH, 28 ºC, 0.21 vvm of air, magnetic stirring at 1000 rpm. ADH99 was used in its soluble form and the activity was fixed at 52.3 U mL^{-1} reaction, corresponding to the maximum loading capacity of the best immobilised derivative obtained (ADH99-epoxy-agarose-UAB M2). Samples (50 µL) were taken periodically and analysed for product quantification as described in Section 3.3.2. Pulses of 10 µL of antifoam were added when foams appeared. The selected process metrics to be optimised were reaction yield (%), Space time yield (STY) (g P L^{-1} h^{-1}) and biocatalyst yield (BY) (mg P mg^{-1} Biocatalyst). STY and BY responses were transformed to *natural log* values using logarithmic regression to normalise the variability of the residual values.

A multiple response optimisation based on a Box-Behnken design (BBD) was used to study the influence of the selected parameters. Substrate concentration, enzyme ratio and reaction time ranges were defined between 100-300 mM, 1.5-1.25 and 2-10 hours, respectively. The limits were estimated keeping in mind previous preparative experiments (data not shown). Each variable was set linearly to 3 levels (-1, 0, 1) as indicated in **Table 4.3**. The data obtained for each response were fitted to a second-order polynomial equation by the least squares method (Eq 1).

$$Y = \beta_0 + \sum \beta_i \cdot X_i + \sum \beta_{ij} \cdot X_i \cdot X_j + \sum \beta_{ii} \cdot X_i^2 + \varepsilon \qquad (1)$$

Where Y is the response variable, β_0 is the offset term, β_i, β_{ij} and β_{ii} are the linear, interaction and quadratic coefficients respectively, and ε is the experimental error. The functions obtained were used to predict the optimal values of the independent variables with Design-Expert 11 software.

In order to find a single optimal condition combining the different responses, optimisation was applied following these criteria: Yield must be maximum and higher than 90 %, STY and BY must be maximised.

3.3.8.2 Statistical analysis

Statistical analyses were performed with Design-Expert 11 software. The quality of fit of the different response surfaces was evaluated with the R^2 and adjusted R^2 coefficients. The significance of the functions and the individual coefficients was determined by analysis of variance (ANOVA) F-test. The lack of fit (LOF) test was used to evaluate differences between experimental and pure error of the fitted equations. In all analyses, p values of 0.05 and 0.1 were considered statistically significant with 95 % and 90 % confidence, respectively.

3.3.8.3 Verification experiment for chlorolactone synthesis reactions with soluble and ADH99-epoxy-agarose-UAB M2 under optimum predicted conditions

Reactions were performed in a 10 mL (reaction volume) tubular mini-reactor as follows: 50 mM potassium phosphate, pH 6.0 (controlled by automatic titration with 1 M NaOH), 1.3 mM NADPH, 28 °C, 0.21 vvm of air, magnetic stirring at 1000 rpm. Substrate concentration, ADH99:NOX ratio and reaction time were those obtained in the optimisation by DoE: 197.4 mM, 1.17 and 6.62 hours, respectively. 52.3 U mL^{-1} reaction of soluble ADH99 or 10 % (v v^{-1}) of immobilised derivative of ADH99-epoxy-agarose-UAB M2 (523 U mL^{-1} support) were used. NOX was added always in soluble form. Antifoam was added when foams appeared. In order to follow the reaction, samples (50 µL) were taken periodically for product quantification as described in Section 3.3.2. Experiments were performed in duplicate. When reusability of the immobilised derivative ADH99-epoxy-agarose-UAB M2 was tested several cycles of reactions were performed using the same described conditions. At the end of each cycle, the immobilised derivative was washed with 100 mL reaction buffer (50 mM potassium phosphate, pH 6.0). Immediately, the same quantities of fresh reaction components used in the previous cycle were added to the recycled immobilised enzyme to start a new reaction cycle. All the cycles were performed over the same reaction time. Experiments were performed in duplicate.

3.3.9 Target reaction: Vanillyl alcohol oxidation by EUGO

3.3.9.1 Vanillin Synthesis Reactions with soluble and immobilised EUGO at 10 mL reaction volume

Reactions with immobilised EUGO were performed using 10 % (v v^{-1}) of the immobilised derivative with respect to the total reaction volume, in order to ensure efficient mixing. To compare catalyst performance in reactions, both soluble and immobilised EUGO were used with the same units per mL of reaction.

Reactions were carried out with 10 mL reaction volume using the following conditions: 400 mM of vanillyl alcohol, 30 % (v v^{-1}) acetone in 50 mM potassium phosphate buffer at pH 7.5, 25 °C, 1 vvm air (hydrated with a 30 % acetone solution in water), magnetic stirring (500 rpm), and 10 µL antifoam. 9 mg mL^{-1} (35847 U mL^{-1} reaction) of catalase were added to the reaction to control the peroxide formed by EUGO in the oxidation. Units of EUGO employed were determined as explained above (Section 3.3.2). Reaction completion time was determined as the moment that no substrate consumption or product production was observed, monitored by GC as described in Section 3.3.10.2.

3.3.9.2 Reusability of the immobilised derivative at 10 mL reaction volume

Several cycles of reactions were performed using the same conditions as in Section 3.3.9.1 using immobilised EUGO as catalyst. At the end of each cycle, the immobilised catalyst was washed three times with 20 mL of water and three times with 20 mL of reaction buffer

(30 % acetone, 50 mM potassium phosphate, pH 7.5). Immediately, the same quantities of fresh reaction components used in the previous cycle were added to the recycled immobilised enzyme to start a new reaction cycle. All the cycles were performed during the same time. The first cycle (using fresh biocatalyst) was terminated upon reach completion and the conversion was determined by GC as shown in Section 3.3.10.2. The reaction time for the rest cycles was the same as for the first one. Error bars correspond to standard deviation.

3.3.9.3 Vanillin synthesis reactions with soluble and immobilised EUGO at 250 mL reaction volume

Reactions with immobilised EUGO were performed using 10 % (v v^{-1}) of the immobilised derivative in order to ensure efficient mixing. To compare catalyst performance in reactions, both soluble and immobilised EUGO were used with the same units of enzyme activity per mL of reaction mixture.

Scaling up of the reactions previously performed [101], was carried out at 250 mL of total reaction volume using the following conditions (high stability **reaction conditions A**): 400 mM of vanillyl alcohol was dissolved in 30 % (v v^{-1}) acetone in 50 mM potassium phosphate buffer, pH 7.5, the mixture agitated by mechanical stirring (1000 rpm) and the temperature adjusted to 25 ºC. Catalase (35847 U mL^{-1}) and EUGO (18 U mL^{-1}) were then added and a stream of acetone saturated air introduced at a rate of 1 vvm. The reaction pH was maintained at pH 7.5 by the controlled addition of 1 M aqueous NaOH using a pH stat and foams were controlled adding pulses of 50 μL of antifoam 1:100 (v v^{-1}) in water. Samples were extracted and analysed over the reaction course and the reaction completion time was defined as the moment that no substrate consumption or product production was observed, monitored by HPLC as described in Section 3.3.10.3. Error bars correspond to standard error.

The reactions with the new conditions tested and optimised by Straatman *et al.* [102] (high activity **reaction conditions B**) were performed by broadly the same procedure shown above, but using 330 mM of vanillyl alcohol in 50 mM glycine-NaOH buffer, pH 9.5 maintained by controlled addition of 5 M NaOH using a pH stat, 25 ºC, 1 vvm air (hydrated with water) mechanical stirring (1000 rpm) and the foams were controlled adding pulses of 50 μL of antifoam. 440 mM of Na_2SO_3 were added to the reaction to eliminate the hydrogen peroxide produced.

EUGO was added to each of the above mixtures to a final concentration of 18 U mL^{-1}. Units of EUGO employed corresponded to the maximum units that could be added to the reaction mixture when immobilised derivatives as optimised in Chapter 5 were used [101].

3.3.9.4 Reusability of the immobilised derivative at 250 mL reaction volume

Several cycles of reactions were performed using the same conditions as in Section 3.3.9.3 using immobilised EUGO as catalyst. At the end of each cycle, the immobilised catalyst was washed with 500 mL reaction buffer (30 % acetone in 50 mM potassium phosphate, pH 7.5 or 50 mM glycine-NaOH buffer, pH 9.5). Immediately, the same quantities of fresh reaction components used in the previous cycle were added to the recycled immobilised enzyme to start a new reaction cycle. All cycles were performed over the same reaction time. The first cycle was ended on reaction completion (using fresh biocatalyst) and the conversion was determined by HPLC as shown in Section 3.3.10.3. The reaction time used for the rest of the cycles was the same as the first one. Error bars correspond to standard error.

3.3.9.5 Vanillin extraction for NMR analysis

A reaction performed under conditions B, was used to purify and crystallise the vanillin as follows: the reaction mixture (250 mL) (100 % conversion and 88 % yield) including a water rinse (50 mL) of the reactor was acidified with 20 % (v v^{-1}) HCl (12 M) to pH 3.0. Isopropyl acetate (250 mL) was added and the mixture heated to 35 ºC for 60 min with stirring. Dicalite 4208 (10 g) was added, briefly mixed, filtered over a precoated glass filter and both phases were separated. The filter cake was washed three times with 125 mL isopropyl acetate. Additional extraction of the water layer was performed with the filter washes (35 ºC, 60 min) and both phases were separated. Finally, the organic layers were combined and the solvent removed by distillation under reduced pressure to afford the product as a white solid. The obtained product was analysed by ^1H and ^{13}C NMR 600 MHz in $CDCl_3$.

3.3.10 GC and HPLC analysis

3.3.10.1 GC analysis of chlorolactone

Chlorolactone quantification was carried out by gas chromatography. Reaction samples (50 µL) were diluted (1:2-1:6 v v^{-1}) in reaction buffer (50 mM potassium phosphate, pH 6.0) and extracted with ethyl acetate (1:20 v v^{-1}) containing 0.1 mg mL^{-1} phenylcyclohexane as internal standard. These samples were centrifuged (13400 rpm, 25 ºC, 2 min) to remove precipitates. The organic phase was filtered with filters of 45 µm and the samples were analysed on an Agilent (7890B) gas chromatography (GC) with a FID detector. A HP5 column with dimensions of 30 m x 320 µ (ID) x 0.25 µm of thickness was used. 5 µL of samples was injected onto the column by split injection (split ratio 20:1) via inlet, which was held at 250 ºC. Helium was used as a carrier gas at constant pressure of 10 psi. Calibration curve is shown in Appendix **Figure 8.1**.

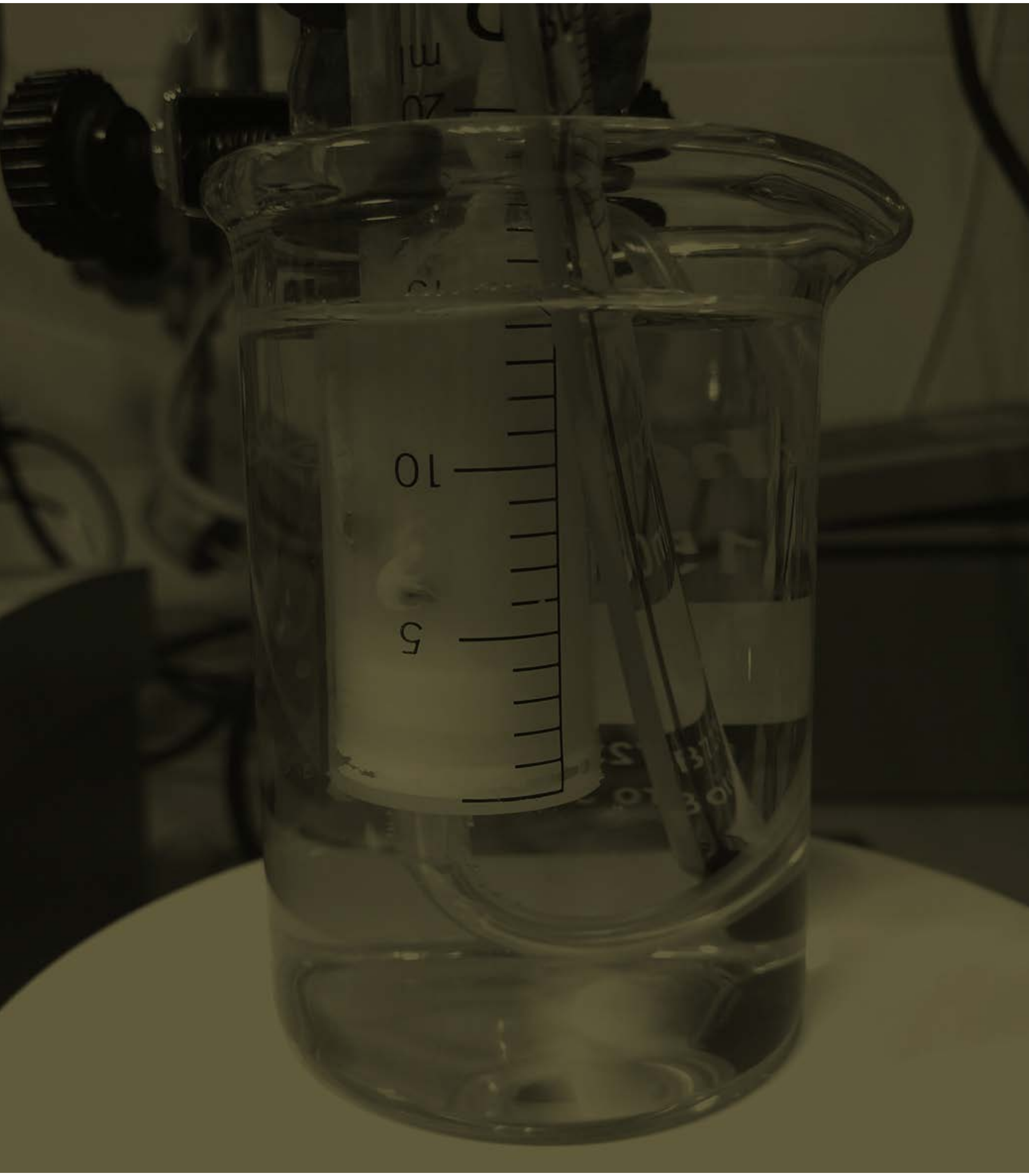
Temperature program was set as follows: initial temperature 100 ºC (0 min), gradient 30 ºC min^{-1} until 250 ºC (3 min). Compounds were detected on a FID detector at 300 ºC. Make-up flow rate was nitrogen gas at constant flow (column + make-up) at 30 mL min^{-1}. Retention times of Internal standard (phenylcyclohexane), chlorolactol and chlorolactone were 3.8, 4.0 and 4.8 min, respectively.

3.3.10.2 GC analysis of vanillyl alcohol and vanillin

Vanillyl alcohol and vanillin quantification, measured in Chapter 5, in preparative scale reactions shown above was carried out by gas chromatography. Samples were extracted with ethyl acetate (1:20 v v^{-1}) containing methylbenzoate (5 mM) as internal standard. The organic phase was analysed using a 7890A Agilent gas chromatograph equipped with an Innowax 19095N-123 (30m x 530 µm x 1 µm) column. The column temperature was held at 100 ºC for 5 min, then increased to 240 ºC at 10 ºC per minute and maintained at this temperature for a further 2 minutes. The injector was kept at 225 ºC; for the flame ionisation detector, the temperature was 250 ºC. Helium was used as a carrier gas at constant pressure of 10 psi. Retention times were 6.9, 17.7 and 19.6 for internal standard, vanillin and vanillyl alcohol, respectively. The calibration curves are shown in Appendix, **Figure 8.2** and 8.3.

3.3.10.3 HPLC analysis of vanillyl alcohol and vanillin

Vanillyl alcohol and vanillin concentrations, measured in Chapter 6, were analysed by HPLC using a Dionex UltiMate 3000 with variable wavelength detector. The reversed-phase column CORTECS TM C18 2.7 µm 4.6x150 mm from Waters (Milford, MA, USA) was employed. Reaction samples were diluted 1:10 (v v^{-1}) in reaction buffer and then further diluted with methanol 1:40 (v v^{-1}) which deactivates the enzyme and stops the reaction. 15 µL of sample were injected in a 0.7 mL min^{-1} mobile phase flow and the column was kept at 30 ºC. All separations were performed by injecting 15 µL of sample at a flow rate of 0.7 mL min^{-1}, 30 ºC. The solvent system consisted of solvent A (0.1% (v v^{-1}) TFA in H_2O) and solvent B (0.095 % (v v^{-1}) TFA in MeCN:H_2O 4:1 (v v^{-1})). Samples were eluted using a gradient from 5 to 15 % B in 0.5 min, then it was increased to 75 % B during 7.5 min, after that, it was increased to 100 % B during 0.5 min and maintained for 2.5 min. Finally, the gradient was decreased to 5 % B during 0.5 min and maintained by 3.5 min to equilibrate the column for the next analysis. A wavelength of λ=231 nm was used to detect the analytes. Prior calibration with standards of known concentration was used for quantitative analysis of all compounds (Appendix, **Figure 8.4** and 8.5). The standard deviation was calculated from duplicated measurements from a single sample. Retention times were 4.9 and 6.7 for vanillyl alcohol and vanillin, respectively.


10
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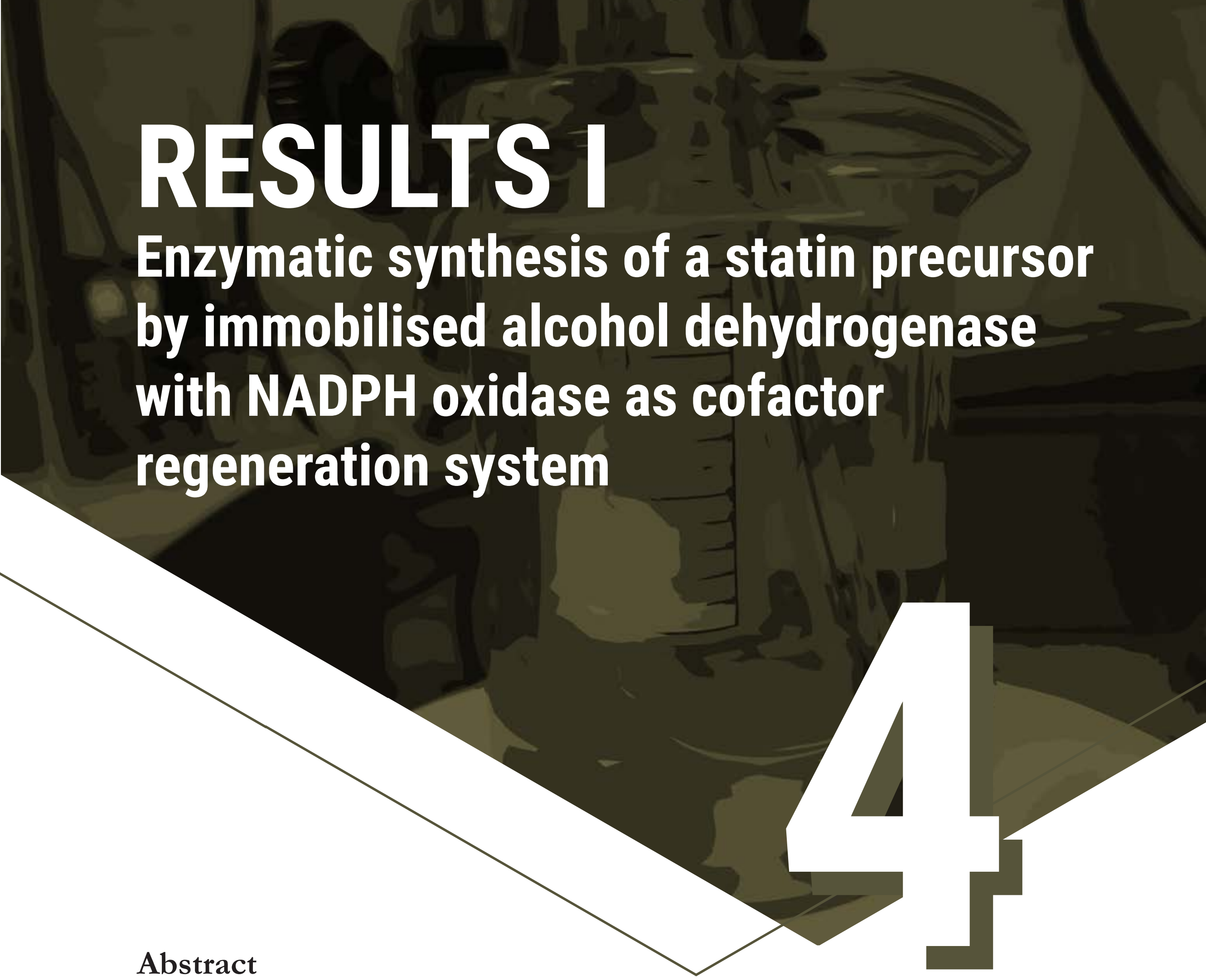


RESULTS I

Enzymatic synthesis of a statin precursor by immobilised alcohol dehydrogenase with NADPH oxidase as cofactor regeneration system

4

Abstract

Statins inhibit the synthesis of LDL-cholesterol which is related to cardiovascular diseases. One of the key steps in the synthesis of the chiral side chain of some statins is the oxidation of chlorolactol to chlorolactone. This oxidation has been performed by an alcohol dehydrogenase (ADH99) using a NADPH-oxidase (NOX) as a cofactor regeneration system. The reaction conditions were optimised obtaining high reaction yield (94.7 %), space time yield (4.6 g P L^{-1} h^{-1}) and biocatalyst yield (7.9 mg P mg^{-1} B). Both enzymes have been efficiently immobilised onto different supports (Eupergit® CM, amino-agarose, epoxy-agarose-UAB M2, Purolite® ECR8409 and ECR8415). ADH99 showed a stability improvement when immobilised. However, NOX did not show any significant stability enhancement. The most stable ADH99 immobilised derivative, ADH99-epoxy-agarose-UAB M2, was used to perform the oxidation, improving 1.5-fold, both the total amount of product produced and the biocatalyst yield compared to the ADH99 soluble form.

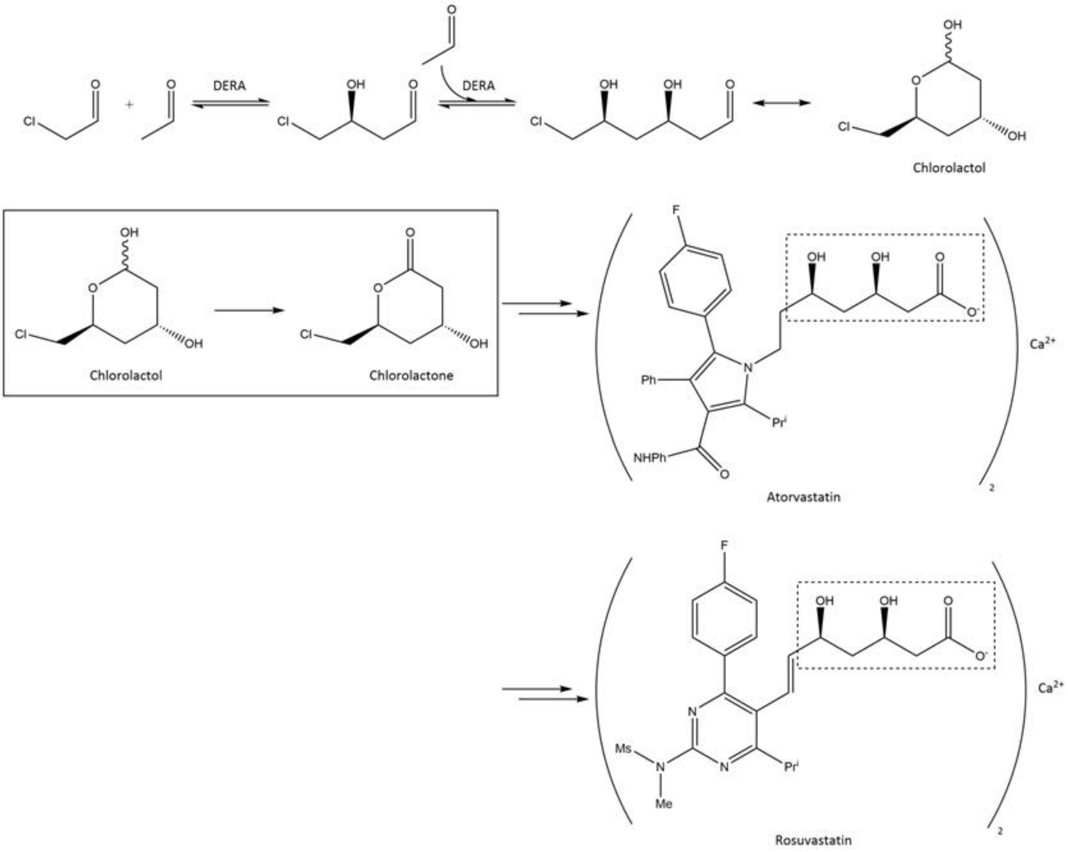
4.1 Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide, with coronary heart disease being the largest contributor [103], [104]. The risk factors are related with sedentary life-style; cigarette smoking, hypertension, obesity, physical inactivity and a high concentration of glucose and cholesterol (mainly low-density lipoprotein (LDL)) in blood [105], [106]. Currently, the treatment of these diseases is focused on prevention, identifying vulnerable patients, and reduction of the risk factors reducing obesity and applying preventive therapies [105].

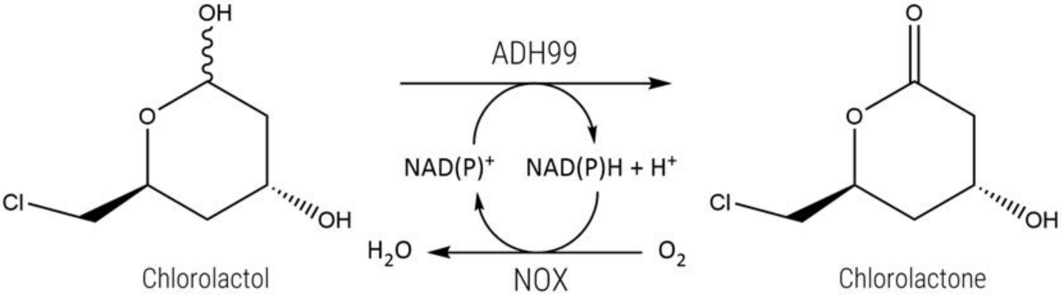
Pharmacological treatments are focused on decreasing LDL cholesterol using drugs such as the statins which act as inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the enzyme that regulates the biosynthesis of cholesterol. There are different types of statins that have been proven as efficient drugs to reduce cholesterol and mortality: i) natural statins, such as Mevastatin and Lovastatin, ii) semisynthetic such as Simvastatin and Pravastatin or iii) synthetic such as Atorvastatin and Rosuvastatin [87], [88], [107]–[109].

In the production of Atorvastatin and Rosuvastatin many approaches have been reported for the biocatalytic synthesis of the chiral 3,5 dihydroxy acid side chain by different routes [90], [110]. A particularly attractive synthetic approach, pioneered by Wong *et al.* is by a multi-step enzymatic pathway using a recombinant 2-deoxyribose-5-phosphate aldolase (DERA) [111] (**Scheme 4.1**). DERA sequentially catalyses the aldol addition of two molecules of acetaldehyde to a two-carbon aldehyde acceptor (chloroacetaldeyde) to give (4*R*,6*S*)-6-(chloromethyl) tetrahydro-2H-pyran-2,4-diol (chlorolactol) [112], [113]. Chlorolactol can then be oxidised to (4*R*,6*S*)-6-(chloromethyl)-4-hydroxytetrahydro-2H-pyran-2-one (chlorolactone) through chemical oxidation with hypoiodite or hypobromite as described in the 1960s [114], or as more recently described in 2004 by reaction with acetic acid and sodium hypochlorite at room temperature [112]. Other chemical oxidations have also been described, such as oxidation with Br_2 and $NaHCO_3$ [115]. In 2015 Jiao *et al.* reported the oxidation using $Ca(ClO)_2$ at 0 ºC [116]. Finally, the chlorolactone could be used as side chain to synthesise Atorvastatin or Rosuvastatin [89].

Only one study has reported the biocatalytic oxidation step from chlorolactol to chlorolactone using lyophilised *E. coli* BL21 whole cells co-expressing ADH (LeADHI87F) and NOX (SmNOXv193R/194H) [89]. However, biooxidation is considered as a route of high interest since biocatalysts allow a high stereoselectivity and stereospecificity, a key factor in this oxidation step. Therefore, biocatalytic methods of oxidising chlorolactol to chlorolactone are of high demand. In the present study, the aforementioned oxidation was performed by an alcohol dehydrogenase (ADH99). The dehydrogenase requires the expensive cofactor NADP$^+$. Aiming to bring the biocatalytic process closer to future industrial implementation, NADPH oxidase (NOX) was used as cofactor regeneration system (**Scheme 4.2**).



Scheme 4.1: Enzymatic synthesis of the chiral side chain of statins catalysed by DERA and chemical synthesis.



Scheme 4.2: Enzymatic oxidation of chlorolactol to chlorolactone catalysed by ADH99 and NOX.

The main goals when using biocatalysts in the synthesis of pharma precursors are to reduce the cost and improve the sustainability, compared to current production processes, through advantages like high enantioselectivity, enantiospecificity, regio-selectivity, reaction rates and stability of the enzyme towards pH, high substrate and solvents concentrations. In addition to the use of low cost raw materials and simple product isolation procedures [117], protein engineering is usually required to reach the desired goals by improving enzyme performance through the improvement of biocatalyst activity and stability. However, other strategies can be an efficient alternative to protein engineering techniques. For example, immobilisation has been extensively reported as a methodology that leads to an improvement in biocatalyst stability, activity, specificity and selectivity, thus enhancing process metrics. Immobilisation also allows the use of different reactor configurations like continuous or cross-flow in addition to traditional batch formats [35], [49], [50].

In the present Chapter, in order to improve the process metrics of chlorolactone synthesis, both enzymes (ADH99 and NOX) were immobilised onto different supports. It should be highlighted that this work is the first report focused on the chlorolactol oxidation to chlorolactone catalysed by isolated and immobilised enzymes which represents a step forward to widen the industrial implementation of enzymatic processes.

4.2 Results and discussion

4.2.1 Characterisation of ADH99 and NOX

ADH99 and NOX, provided by c-LEcta and DSM/InnoSyn, respectively, were characterised prior to any experiment. Protein content and enzymatic activity of both enzymes were measured, resulting in specific activities of 10.8 and 22.5 U mg^{-1} protein, respectively.

Enzymatic activity and stability are key factors to select the optimum immobilisation conditions, the most suitable supports and the optimum reaction conditions [49]. Therefore, the effect of pH on the activity and stability was studied for both, ADH99 and NOX (**Figure 4.1** and **4.2**). ADH99 has maximum activity at pH 5.5 while NOX at pH 6.5. Regarding enzyme stability, ADH99 preserved good stability in the range of pH 6.0 to 8.5 with optimum activity observed between pH 7.0 and 8.0 where it maintained more than 80 % of its initial activity after 48 h (**Figure 4.2** (**A**)). On the other hand, NOX showed lower stability than ADH99, with 36 % of its initial activity remaining after 48 h of incubation at its optimal pH of 6.5 (**Figure 4.2** (**B**)).

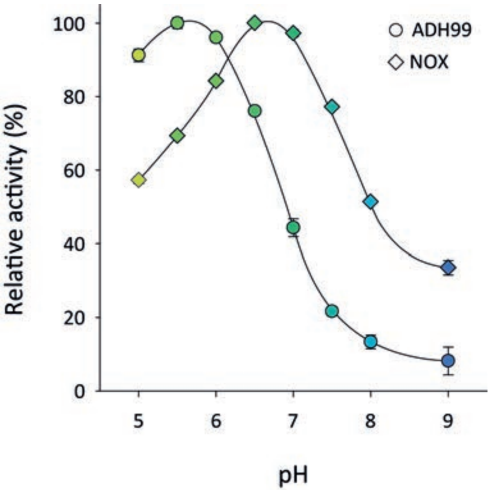


Figure 4.1: pH activity profile for ADH99 and NOX. Substrate solution was prepared in the following 100 mM buffers: sodium acetate (pH 5.0 and 5.5), potassium phosphate (pH 6.0-8.0) and Tris-HCl (pH 9.0), 30 °C. Error bars correspond to the standard error of two replicates.

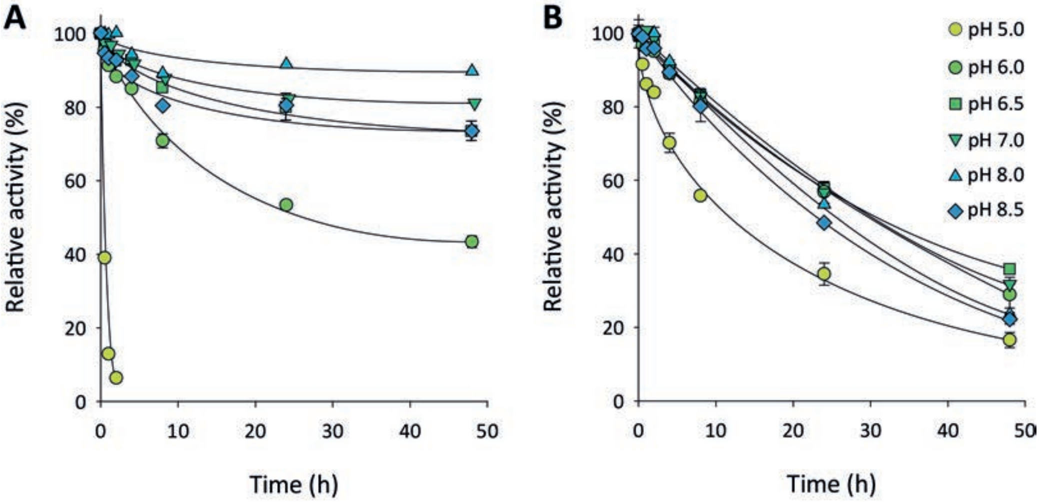


Figure 4.2: Effect of pH on ADH99 (**A**) and NOX (**B**) stability. Stability was studied by incubating 1 U mL^{-1} of enzyme in 100 mM buffers: sodium acetate (pH 5.0), potassium phosphate (pH 6.0-8.5), 28 ºC. Error bars correspond to the standard error of two replicates.

4.2.2 Immobilisation of the enzymes

Different supports, with different matrices and functional groups were selected to immobilise ADH99 and NOX aiming to improve their stability: methacrylate matrix supports functionalised with amino (Purolite® ECR8409 and Purolite® ECR8415) and epoxy groups (Eupergit® CM), and agarose matrix supports functionalised with amino (amino-agarose) and epoxy groups (epoxy agarose-UAB M2).

In order to characterise the immobilisations, first of all, low loadings of enzyme were offered to the selected supports in order to ensure that diffusional limitations were not observed. Different concentrations of enzyme were offered to the supports (2.5-20 U mL^{-1} support). No diffusional limitations were observed, showing same immobilisation and retained activities, when the offered activity was equal or lower than: 20, 5, 10, 10 and 10 U ADH-99 mL^{-1} support or 9, 12, 10, 10 and 10 U NOX mL^{-1} support for amino-agarose, Eupergit® CM, epoxy-agarose-UAB M2, Purolite® ECR8409 and Purolite® ECR8415, respectively.

Once ensured that no diffusional limitations were observed, optimisation of the immobilisation was performed for ADH99.

Immobilisation on amino-functionalised supports (amino-agarose, Purolite® ECR8409 and Purolite® ECR8415) occurs in two-steps: an ionic adsorption followed by covalent bonding. In the second step CDI is added as carboxyl activating agent to promote amide bond formation between the amine groups from the support and carboxyl groups of the enzyme. Immobilisation has to be carried out at pH conditions higher than the pI of the enzyme to negatively charge it and lower than the support pKa (6.8 for amino-agarose [118] and 10.7 for Purolite® supports), to positively charge it. Thus, pH 6.5 was selected as a suitable pH to perform the immobilisation, taking into account both, the pKa of the support and the stability results (**Figure 4.2**).

Regarding ADH99 immobilisation on amino-agarose, the ionic adsorption was completed after 30 min. Different CDI concentrations were studied obtaining 11 ± 1, 20 ± 0, and 25 ± 1 % retained activities for 50, 25, and 10 mM CDI respectively. 10 mM CDI was the concentration that produced the best results for ADH99 immobilisation onto amino-agarose (**Table 4.1**, **row 4**). These optimised immobilisation conditions are similar to those reported by Solé *et al.* [60] when immobilising an alcohol dehydrogenase on the same support. However, immobilisation of ADH from *Artemisia annua* resulted on slightly lower retained activity (13.2 %) compared to the 26 ± 1 % obtained for the ADH99 in the present study. These optimised conditions were used to immobilise ADH99 onto the Purolite® ECR8409 and Purolite® ECR8415, reaching low retained activities (<10 %) (**Table 4.1**, **rows 1 and 2**). In this case, Solé *et al.* obtained a higher retained activity with Purolite® ECR8409 using a similar method (20.2 %).

Immobilisation of NOX was also studied on amino-functionalised supports. Regarding amino-agarose, 50, 25 and 10 mM of CDI were tested achieving 37 ± 2, 36 ± 1 and 28 ± 1 % of retained activities, respectively. 50 mM was selected as the optimum (**Table 4.1**, **row 9**). Immobilisation of NOX on Purolite® ECR8409 and Purolite® ECR8015 was also performed under the same conditions than amino-agarose (**Table 4.1**, **row 6 and 7**) reaching 23 and 21 % retained activities.

Table 4.1: ADH99 and NOX Immobilisation screening.

#	Enzyme	Matrix	Support	Support functional group	U offered mL^{-1} support	pH	IY (%)	RA (%)
1	ADH99	Methacrylate	Purolite® ECR8409	Amino	10	6.5	99 ± 1	8 ± 1
2			Purolite® ECR8415	Amino	10	6.5	99 ± 0	5 ± 1
3			**Eupergit® CM**	**Epoxy**	**5**	**8.0**	**96 ± 2**	**55 ± 3**
4		Agarose	**Amino-agarose**	**Amino**	**20**	**6.5**	**94 ± 1**	**26 ± 1**
5			**Epoxy-agarose-UAB M2**	**Epoxy**	**10**	**8.0**	**100 ± 2**	**62 ± 2**
6	NOX	Methacrylate	Purolite® ECR8409	Amino	10	6.5	97 ± 3	23 ± 1
7			Purolite® ECR8415	Amino	10	6.5	96 ± 7	21 ± 1
8			**Eupergit® CM**	**Epoxy**	**12**	**8.0**	**100 ± 1**	**35 ± 2**
9		Agarose	**Amino-agarose**	**Amino**	**9**	**6.5**	**100 ± 0**	**37 ± 2**
10			**Epoxy agarose-UAB M2**	**Epoxy**	**10**	**8.0**	**71 ± 0**	**21 ± 1**

IY: immobilisation yield, RA: retained activity.

Immobilisations onto supports with epoxy-groups (Eupergit® CM and epoxy-agarose-UAB M2) are usually carried out in a neutral or alkaline pH buffer solution since the opening of epoxy rings, resulting in covalent bond formation with the amino groups of the enzyme, is more favourable as pH is increased because a higher proportion of amine residues are present in an uncharged form [119], [120][121]. However, strong alkaline conditions which promote a faster covalent binding formation cannot be applied due to the low enzyme stability. Therefore, immobilisation of ADH99 and NOX onto epoxy-functionalised supports were performed at pH ≥7.0 taking into account the stability issues. Immobilisation of ADH99 was performed on Eupergit® CM at pH 8.0 during different incubation times (1, 2, 4 and 21 h). Results revealed that at pH 8.0, almost complete immobilisation takes place reaching retained activities of 34 ± 2, 47 ± 1, 55 ± 3 and 47 ± 1 % after 1, 2, 4 and 21 h respectively. Maximum retained activity was obtained after 4 h of incubation. Lower values were obtained after 21 h due to a decay in enzyme activity, probably because of additional covalent attachment formation over longer times. Immobilisation of ADH99 was also tested at pH 7.0 over 4 h, giving 32 ± 1 % retained activity. These results were expected since decreasing pH leads to slower covalent bond formation. Therefore, pH 8.0 was selected as the most suitable pH for ADH99 immobilisation on Eupergit® CM (Table 4.1, **row 3**). The optimised conditions obtained for Eupergit® CM, pH 8.0 and 4 h, were the conditions used to immobilise ADH99 onto epoxy-agarose-UAB M2, reaching 62 ± 2 % retained activity (Table 4.1, **row 5**). These results are in accordance with similar immobilisations of other ADHs by Solé, *et al.* who reported 58 % retained activity for an ADH from *Artemisia annua* immobilised onto epoxy agarose [60]. Other authors have also reported high retained activity values (up to 68 %) for other ADHs and oxidoreductases when immobilised on epoxy functionalised supports [60] [16], [63].

NOX immobilisation onto Eupergit® CM and epoxy-agarose-UAB M2 leads to retained activities of 35 ± 2 and 21 ± 1 %, respectively (Table 4.1, **row 8 and 10**). However, when epoxy-agarose-UAB M2 was used only a 71 ± 0 % immobilisation yield was reached.

Among all the supports studied, Eupergit® CM, amino-agarose and epoxy-agarose-UAB M2 were selected due to the highest retained activities obtained for ADH99, the main enzyme performing the oxidation of chlorolactol to chlorolactone. (Table 4.1, **rows 3, 4 and 5**). The selected immobilised derivatives are in bold in the table.

4.2.3 Stability under reaction and storage conditions of ADH99 and NOX in soluble form and when immobilised on Eupergit® CM, amino-agarose and epoxy-agarose-UAB M2

One of the advantages of enzyme immobilisation is stability improvement [51]. Therefore, the stabilities of soluble and immobilised ADH99 and NOX were studied under reaction conditions (50 mM potassium phosphate pH 6.0, 28 ºC, mild agitation conditions) and storage conditions (50 mM potassium phosphate pH 6.5, 4 ºC). The pH selected for the storage conditions was set, aiming to apply a compromise pH where both enzymes could be stored together, if necessary. According to Figure 4.2, ADH99 preserved good stability in the range of pH 6.0 to 8.5 and NOX, which is the most unstable enzyme, showed its maximum stability below pH 6.5. Therefore, storage conditions were set at pH 6.5. As shown in Figure 4.3 (**A**), using the reaction conditions defined above, soluble ADH99 was found to have a half-life of 9 h, whereas ADH99 immobilised on Eupergit® CM, amino-agarose and epoxy-agarose-UAB M2, improved half-life by 13.7, 8.9 and 3.7-fold respectively. These results fit with other improvements in ADH stability through immobilisation towards different parameters such as salt concentration [122], cosolvents [123], temperature and pH when immobilised [124]–[126].

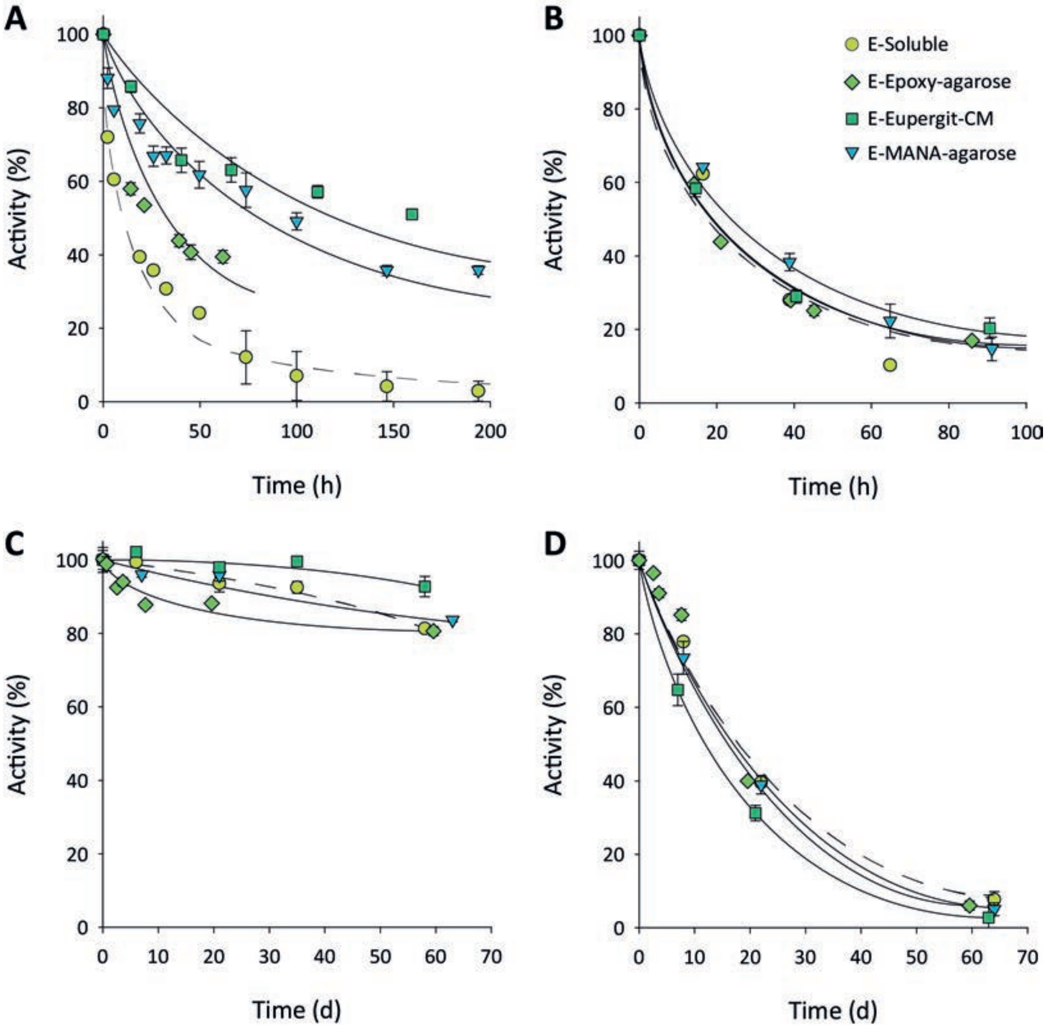


Figure 4.3: Stability of ADH99 (**A**) and NOX (**B**), soluble and immobilised onto the selected supports, under reaction conditions (50 mM potassium phosphate pH 6.0, 28 °C) and under storage conditions (50 mM potassium phosphate pH 6.5, 4 °C) ADH99 (**C**) and NOX (**D**), respectively. Error bars correspond to the standard error of two replicates.

However, no improvement was observed with any of the NOX immobilised derivatives when compared with the soluble form, all showing half-lives of 18 h (Figure 4.3 (**B**)). Stability under storage conditions of all the immobilised derivatives of both enzymes did not show improvement when compared with soluble forms Figure 4.3 (**C-D**).

4.2.4 Maximum enzyme loading on Eupergit® CM, amino-agarose and epoxy-agarose UAB M2

The characterisation of enzyme immobilisation at low loadings allows the determination of enzyme activity per unit of support (retained activity) in the absence of diffusional limitations (Table 4.1). Assuming that the percentage of theoretical retained activity at both low and high loadings will be similar, the retained activity value obtained in the absence of diffusional limitations can be used to predict the theoretical retained activity at high loadings, where diffusional limitations exist. Thus, the values obtained at low loadings were used to calculate the theoretical final activity of the high-loaded derivatives. In order to optimise the maximum enzyme loading capacity of each enzyme for each support, different loadings of enzyme were offered to them (Table 4.2). Almost all results do not show a limit of the maximum retained activity value which may indicate multi-layer immobilisations. However, at a given loading, the immobilisation yield drastically decreases leading to a high loss of activity in the supernatant. The loading prior to this decrease was selected as the maximum enzyme capacity, preventing unnecessary high enzyme losses.

Taking into account the retained activities of ADH99 immobilised onto Eupergit® CM (55 ± 3 %), epoxy-agarose-UAB M2 (62 ± 2 %) and amino agarose (26 ± 1 %), the Eupergit® CM and the epoxy-agarose-UAB M2 derivatives showed the highest activities of 414 ± 1 and 523 ± 1 U mL^{-1} support, without high enzyme amount lost in the supernatant, respectively. amino-agarose derivative, however, showed less than 10-fold retained activity reaching 34 ± 0 U mL^{-1} support (in bold in Table 4.2).

Regarding NOX, taking into account the retained activities obtained at low loads (35 ± 2 %

Table 4.2: Results of maximum enzyme loading immobilised on the selected supports, following the procedures described in sections 3.3.5.1, 3.3.5.2 and 3.3.7. Experiments were performed by duplicate.

#	Enzyme	Support	U offered mL^{-1} support	% immobilisation	theorical U immobilised mL^{-1} support	Theoretical retained activity (U mL^{-1} support)	Measured activity of the immobilised derivative (U mL^{-1})
1	ADH99	Eupergit® CM (RA: 55%)	545	98 ± 0	531 ± 0	294 ± 0	10 ± 4
2			**817**	**92 ± 0**	**748 ± 2**	**414 ± 1**	**14 ± 3**
3			1089	76 ± 1	824 ± 14	456 ± 8	19 ± 2
4			2180	48 ± 1	1036 ± 21	573 ± 12	33 ± 5
5		Epoxy-agarose-UAB M2 (RA: 64%)	172	99 ± 0	171 ± 0	109 ± 0	16 ± 2
6			345	99 ± 0	340 ± 0	218 ± 0	23 ± 3
7			517	99 ± 0	511 ± 0	327 ± 0	25 ± 0
8			690	98 ± 0	676 ± 0	432 ± 0	30 ± 1
9			**862**	**95 ± 0**	**817 ± 2**	**523 ± 1**	**33 ± 0**
10		Amino-agarose (RA: 26%)	**164**	**81 ± 0**	**133 ± 0**	**34 ± 0**	**6 ± 2**
11			555	71 ± 1	396 ± 7	101 ± 2	13 ± 0
12			833	60 ± 0	498 ± 3	127 ± 1	13 ± 4
13			1110	47 ± 1	525 ± 14	134 ± 3	15 ± 5
14			2222	28 ± 0	620 ± 9	158 ± 2	14 ± 2
15	NOX	Eupergit® CM (RA: 36%)	**1045**	**83 ± 0**	**865 ± 3**	**309 ± 1**	**71 ± 5**
16			1567	65 ± 0	1013 ± 0	362 ± 0	91 ± 11
17			2089	47 ± 1	982 ± 17	350 ± 6	89 ± 2
18			4179	31 ± 1	1305 ± 50	466 ± 18	84 ± 5
19		Epoxy-agarose-UAB M2 (RA: 21%)	603	94 ± 0	569 ± 1	268 ± 0	18 ± 4
20			**1207**	**93 ± 0**	**1121 ± 0**	**332 ± 0**	**20 ± 5**
21			1810	77 ± 0	1395 ± 2	413 ± 0	27 ± 3
22			2413	68 ± 0	1639 ± 10	485 ± 1	31 ± 0
23			3017	60 ± 0	1810 ± 15	535 ± 2	31 ± 0
24		Amino-agarose (RA: 37%)	1051	94 ± 0	983 ± 1	365 ± 0	78 ± 6
25			**1576**	**80 ± 0**	**1261 ± 4**	**468 ± 2**	**91 ± 8**
26			2101	55 ± 1	1148 ± 14	426 ± 5	91 ± 11
27			4202	20 ± 1	822 ± 57	305 ± 21	46 ± 2

for Eupergit® CM, 21 ± 1 % for epoxy-agarose-UAB M2 and 37 ± 2 % for amino-agarose), the highest activity was obtained with the amino-functionalised support (468 ± 2 U mL^{-1} support). Eupergit® CM and epoxy-agarose-UAB M2 showed very similar final theoretical activities: 309 ± 1, 332 ± 0 U mL^{-1} support respectively (**Table 4.2**). The selected loads are in bold in the table. As expected, the measured activity of the immobilised derivatives was lower than the theoretical retained activity due to the diffusional limitations. ADH99 immobilised derivatives showed between 6 and 33 U mL^{-1} support, compared to 20-91 U mL^{-1} for NOX derivatives.

4.2.5 Optimisation of Chlorolactol oxidation by DoE

Considering that chlorolactone is unstable under aqueous conditions [89], epoxy-agarose-UAB M2 was the support selected as it is the support with the maximum ADH99 loading capacity that will give the fastest reaction, aiming to reduce the reaction time to prevent product degradation. Therefore, reaction condition optimisation was carried out using soluble enzymes with the maximum ADH99 units that could be offered to the reaction when ADH99-epoxy-agarose-UAB M2 is used (i.e. 10 % v v^{-1}; 52.3 U mL^{-1} reaction). Regarding NOX, since its stability could not be improved by immobilisation, it was decided to use it as a soluble enzyme for all experiments.

The main influencing parameters of the reaction conditions (substrate concentration, enzyme ratio and reaction time) were optimised in order to improve the process metrics applying a response surface methodology. Other parameters such as temperature or oxygen were previously optimised by partners of the ROBOX project. An advanced optimisation strategy based on Design of Experiments (DoE) was selected to screen a wide range of the selected parameters. Previous reactions were carried out (data not shown) in order to select the suitable ranges to study in the Box-Behnken design (BBD) (**Table 4.3**). The process metrics evaluated were the reaction yield, STY and BY. The resulting BBD matrix consisted in 15 experiments (**Table 4.3**). The obtained functions were 3D plotted showing the surface-response for each process metric (**Figure 4.4**).

Table 4.3: Box-Behnken design, experimental results for each response and ANOVA analysis.

Box-Behnken design

	Variables			Responses		
Experimental run	**S (mM)**	**r (ratio ADH99:NOX)**	**t (h)**	**Yield (%)**	**STY (g P L^{-1} h^{-1})**	**BY (mg P mg B^{-1})**
1	100	0.5	6	99	2.7	2.9
2	200	0.5	2	62	10.3	3.6
3	200	2	2	73	12.0	7.2
4	100	1.25	2	70	5.8	3.0
5	200	1.25	6	97	5.3	8.4
6	200	1.25	6	100	5.5	8.6
7	300	2	6	70	5.8	10.3
8	200	2	10	74	2.4	7.3
9	300	1.25	2	57	14.1	7.4
10	200	0.5	10	94	3.1	5.5
11	100	1.25	10	94	1.5	4.1
12	300	0.5	6	80	6.6	7.0
13	300	1.25	10	80	4.0	10.4
14	200	1.25	6	98	5.4	8.5
15	100	2	6	100	2.7	4.9

Analysis

Function	F test, p-value	LOF test, p-value	R^2	Adjusted R^2
Yield	0.0002	0.06	0.963	0.927
STY	<0.0001	0.05	0.998	0.995
BY	<0.0001	0.05	0.994	0.987

Model	**Yield (%)**		**STY (g P L^{-1} h^{-1})**		**BY (mg P mg B^{-1})**	
Parameters	**Coefficient**	**p-value**	**Coefficient**	**p-value**	**Coefficient**	**p-value**
Constant	98.3		1.69		2.14	
S	-9.5	<0.01	0.43	<0.01	0.43	<0.01
r	-2.4	0.14	-0.03	0.13	0.24	<0.01
t	10.0	<0.01	-0.67	<0.01	0.13	<0.01
$S \cdot r$	NS	>0.1	-0.03	0.16	NS	>0.1
$r \cdot t$	-7.7	<0.01	-0.10	<0.01	-0.10	<0.01
$S \cdot t$	NS	>0.1	NS	>0.1	NS	>0.1
S^2	-5.9	0.03	-0.22	<0.01	-0.22	<0.01
r^2	-5.2	0.04	-0.06	0.04	-0.19	<0.01
t^2	-17.1	<0.01	0.08	<0.01	-0.21	<0.01

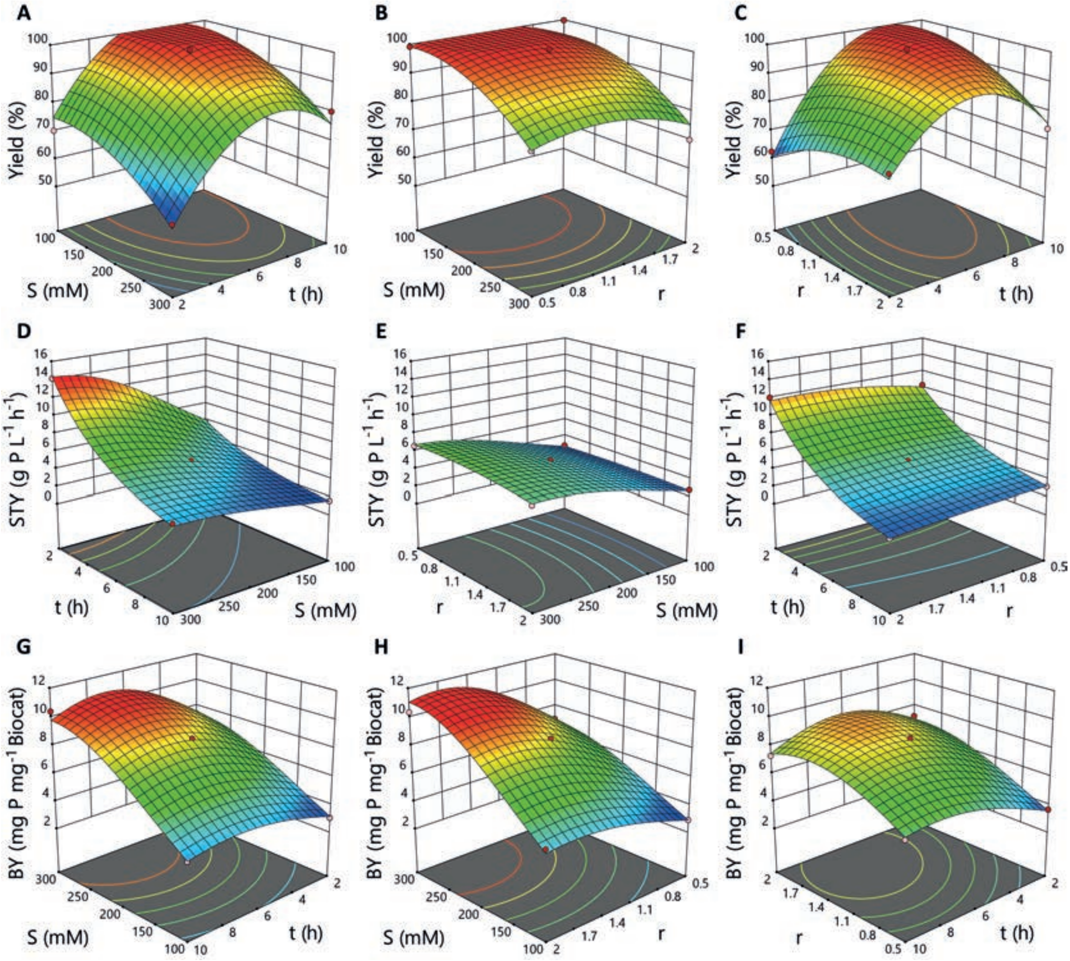


Figure 4.4: Surface plots of the experimental results of the Box-Behnken design. Yield (**A**-**C**), STY (**D**-**F**), BY (**G**-**I**) in function of the variables two variables and letting constant the other one at its average value ratio ADH99:NOX = 1.25 (**A**, **D**, **G**), time = 6 h (**B**, **E**, **H**) and Substrate = 200 mM (**C**, **F**, **I**).

Maximum yields were obtained at low substrate concentrations (100-202 mM), reaction times between 5.3 and 9.2 hours and low ADH99:NOX ratios (0.5-1.2). Higher reaction times lead to a decrease in yield, probably due to product instability. According to the statistical analysis (**Table 4.3**, p-values), substrate, time, the dual effect of time and enzyme ratio were the most significant factors affecting the reaction yield (**Figure 4.4**, **A-C**)).

Maximum STY was obtained at the lowest reaction time (2 h), the highest substrate concentrations (300 mM) and the lowest enzyme ratio (0.5) (**Figure 4.4**, **D-F**). According to the statistical analysis (Table 4.3, p-values), substrate concentration, time, the interaction between the enzyme ratio and time were the most significant factors affecting STY. The quadratic terms of substrate and time show that these responses were non-linear. This non-linear dependence as well as the interdependence between enzyme ratio and time indicate that this reaction optimisation taking one parameter at a time would have been inefficient. Maximum BY was obtained at high substrate concentration (297 mM), 1.7 enzyme ratio and 6.7 h of reaction time. The statistical analysis (Table 4.3, p-value) showed that all 3 factors, the interaction between enzyme ratio and time and between substrate and time as well as the effects of all three parameters, which are non-linear, had the most significant effect on BY.

In order to define the optimal conditions for the target reaction, the following criteria were considered: reaction yield was fixed to be >90 % and as close as possible to 100 %, the STY and BY were fixed to obtain the maximum values. The values of independent variables that optimise the process metrics and the predicted response of the model are shown in Table 4.4. The model was validated performing 4 replicate experiments using the optimal reaction conditions and all the responses fitted in the ranges of confidence.

Table 4.4: Independent variables and predicted responses that optimise the process metrics with the confidence interval for 4-replicate validation.

Optimal parameters values		
S (mM)	**ratio ADH99:NOX**	**Time (h)**
197.4	1.17	6.62

Response	**Predicted response**	**Standard error Predicted**	**95% lower confidence interval**	**95% upper confidence interval**	**Experimental results**
Yield (%)	100	3	93	100	95 ± 2
STY (g P L^{-1} h^{-1})	4.8	N/A	4.5	5.2	4.6 ± 0.1
BY (mg P mg B^{-1})	8.3	N/A	7.6	9.1	7.9 ± 0.1

4.2.6 Chlorolactol oxidation performed by immobilised ADH99-epoxy-agarose-UAB M2 under optimised conditions

Reaction with immobilised ADH99 onto the selected support (epoxy-agarose-UAB M2) and soluble NOX was performed under the same conditions and compared to the performance of using both soluble AD99 and NOX (**Figure 4.5**).

Using soluble ADH99, 308 ± 5 mg of product was obtained with in 95 ± 2 % yield, with a STY of 4.6 ± 0.1 g P L^{-1} h^{-1}, BY of 7.9 ± 0.1 mg P mg^{-1} B and 85 ± 9 mM h^{-1} of initial product formation rate (rp).

When immobilised ADH99 was used, yield, STY and biocatalyst yield were 18 % lower compared to the use of both soluble enzymes. Moreover, rp also decreased 11 % (**Table 4.5**). This decrease observed when immobilised derivative was used could be due to the presence of diffusional limitations and/or due to a lower retained activity than expected due to the assumption that the retained activity using high and low loadings was the same.

Only one study was found in the literature (2016) that reported chlorolactone production by biocatalysis with lyophilised *E. coli* BL21 whole cells co-expressing ADH (LeADHI87F)

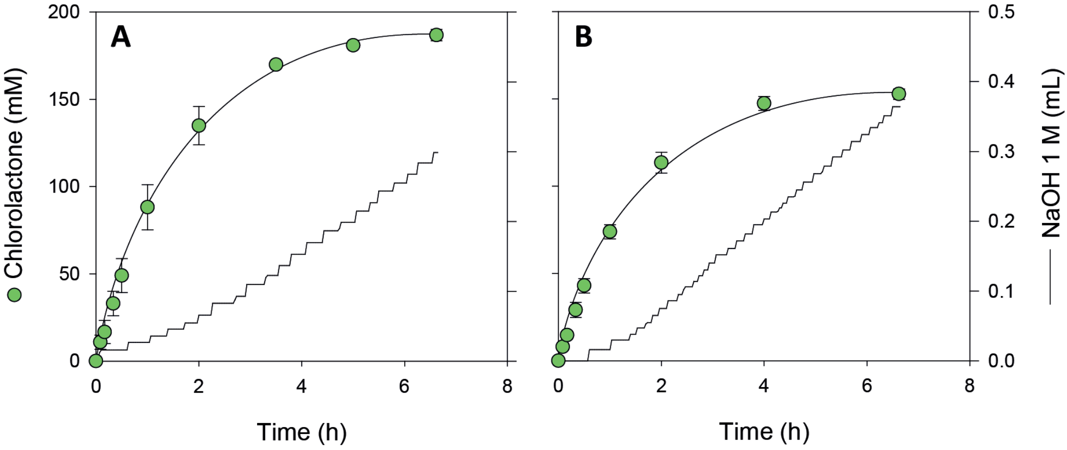


Figure 4.5: Chlorolactone reaction profile of soluble (**A**) and immobilised (**B**) ADH99 (52.3 U mL^{-1} reaction). Reaction conditions: 44.7 U NOX mL^{-1} reaction, 197.4 mM of Chlorolactol in 50 mM potassium phosphate pH 6.0 controlled by titration with 1 M NaOH, 1.3 mM NADPH, 0.21 vvm of air, 28 °C, magnetic agitation (1000 rpm) 10 mL. Reaction time: 6.62 h. Error bars represent the standard error of two replicates.

and NOX (SmNOXv193R/194H) [89]. The authors used 10-20 g L^{-1} of cells to convert 300 mM substrate in 100 mM potassium phosphate at pH 7.0 with 0.1 mM $NADP^+$ over 10 h, obtaining practically the same STY reported in the present work (4.7 g P L^{-1} h^{-1}).

4.2.7 Reusability of ADH99-epoxy-agarose-UAB M2 in chlorolactol oxidation

ADH99-epoxy-agarose-UAB M2 reusability was tested for the chlorolactol oxidation. Three reaction cycles could be accomplished reaching 77 ± 2, 41 ± 2 and 22 ± 0 % yield, respectively (**Figure 4.6**). This allowed a combined mass of 458 ± 0 mg of product to be obtained which represents a 1.5-fold improvement when compared with the soluble reaction. Nevertheless, STY could not be improved: 0.8 ± 0.0 g P L^{-1} h^{-1} of STY was obtained, 6-fold lower than the values obtained with the soluble reaction due to a decrease in enzyme activity. The total biocatalyst yield (7.0 ± 0.0 mg P mg^{-1} B) obtained was also lower (11 %) compared to the soluble reaction (7.9 ± 0.1 mg P mg^{-1} B) (**Table 4.5**). However, considering only the ADH99, which is the biocatalyst that is re-used, BY was improved 1.5-fold when compared to the use of soluble enzyme (11.9 ± 0.2 mg P mg^{-1} ADH99 and 17.8 ± 0.0 mg P mg^{-1} ADH99,

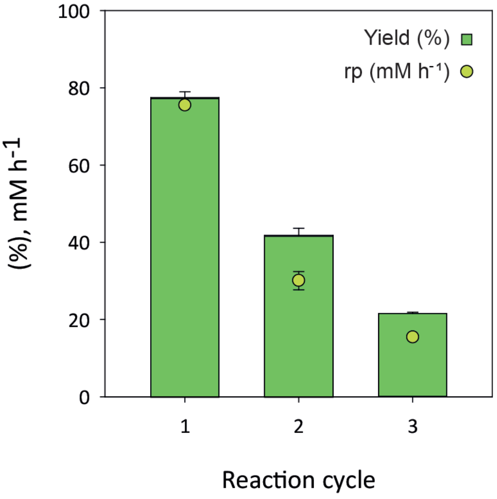


Figure 4.6: Yield and initial production rate (rp) of the reaction cycles. ADH99 immobilised onto epoxy-agarose-UAB 523 U mL^{-1} support (52.3 U mL^{-1} reaction). Reaction cycle: 6.62 h. Reaction conditions: 44.7 U NOX mL^{-1} reaction, 197.4 mM of Chlorolactol in 50 mM potassium phosphate pH 6.0 controlled by titration with 1 M NaOH, 1.3 mM NADPH, 0.21 vvm of air, 28 °C, magnetic agitation (1000 rpm) 10 mL. Error bars represent the standard error of two replicates.

Table 4.5: Results of reactions with soluble and immobilised ADH99 at optimised reaction conditions.

Reaction	yield (%)	STY (g P L^{-1} h^{-1})	BY (mg P mg^{-1} B)	BY (mg P mg^{-1} ADH99)	Total P (mg)	rp (mM h^{-1})
Soluble	95 ± 2	4.6 ± 0.1	7.9 ± 0.1	11.9 ± 0.2	308 ± 5	85 ± 9
Immobilised	77 ± 2	3.8 ± 0.1	6.5 ± 0.1	9.8 ± 0.2	252 ± 5	76 ± 0
Reaction cycles	46 ± 0	0.8 ± 0.0	7.0 ± 0.0	17.8 ± 0.0	458 ± 0	-

respectively). According to these results, the use of both soluble ADH99 and NOX resulted in the best option to perform the synthesis of Chlorolactone. Moreover, the cost of the immobilisation support and immobilisation process is avoided.

Other authors have reported reutilisation of immobilised ADHs. Rulli *et al.* could perform 2 reaction cycles in the reduction of acetophenone with an ADH from *Lactobacillus kefir* [127]. ADH-'A' from *Rhodococcus ruber* DSM 44541 immobilised onto different amino-functionalised supports, derivatised with glutaraldehyde, could be reused to perform 3 reaction cycles in the reduction of acetophenone [128]. Moreover, improvements in biocatalyst yield when ADH is used in immobilised form were also reported. Solé *et al.* reported 4 reaction cycles in the synthesis of ketoisophorone with ADH from *Artemisia annua* improving the biocatalyst yield up to 2.5-fold [60].

4.3 Conclusions

ADH99 and NOX were immobilised efficiently onto different supports (Eupergit® CM, epoxy agarose-UAB M2 and amino-agarose) with high enzyme loadings (414 ± 1, 523 ± 1 and 34 ± 0 U mL^{-1} support for ADH99 and 309 ± 1, 332 ± 0 and 468 ± 2 U mL^{-1} support for NOX, respectively). Stability of ADH99 was strongly improved by immobilisation on all the supports screened compared to the soluble form (3.7 to 13.7-fold). However, no stability improvements were observed for immobilised NOX. Reaction conditions were optimised by DoE aiming to improve the process metrics obtaining high reaction yields (95 ± 2 %), Space-Time yields (4.6 ± 0.1 g P L^{-1} h^{-1}) and Biocatalyst yields (7.9 ± 0.1 mg P mg^{-1} B). These promising values make ADH99 and NOX a good enzymatic system to synthesise the chiral side chain of statins. Moreover, this biocatalytic process represents a greener procedure than the chemical synthesis of chlorolactone since i) hypochlorite, Br_2, $NaHCO_3$ are avoided, ii) cooling step is not required iii) the reaction can be carried out under mild conditions iv) oxygen is the only oxidant required and v) water is produced as sub-product. Immobilised ADH99 could be reused up to 3 reaction cycles improving 1.5-fold both, the total product amount and the biocatalyst yield (mg P mg^{-1} ADH99). Nevertheless, soluble enzymes resulted in the best formulation in terms of process metrics to perform the target reaction. The results of the research of this chapter has recently been published [129].

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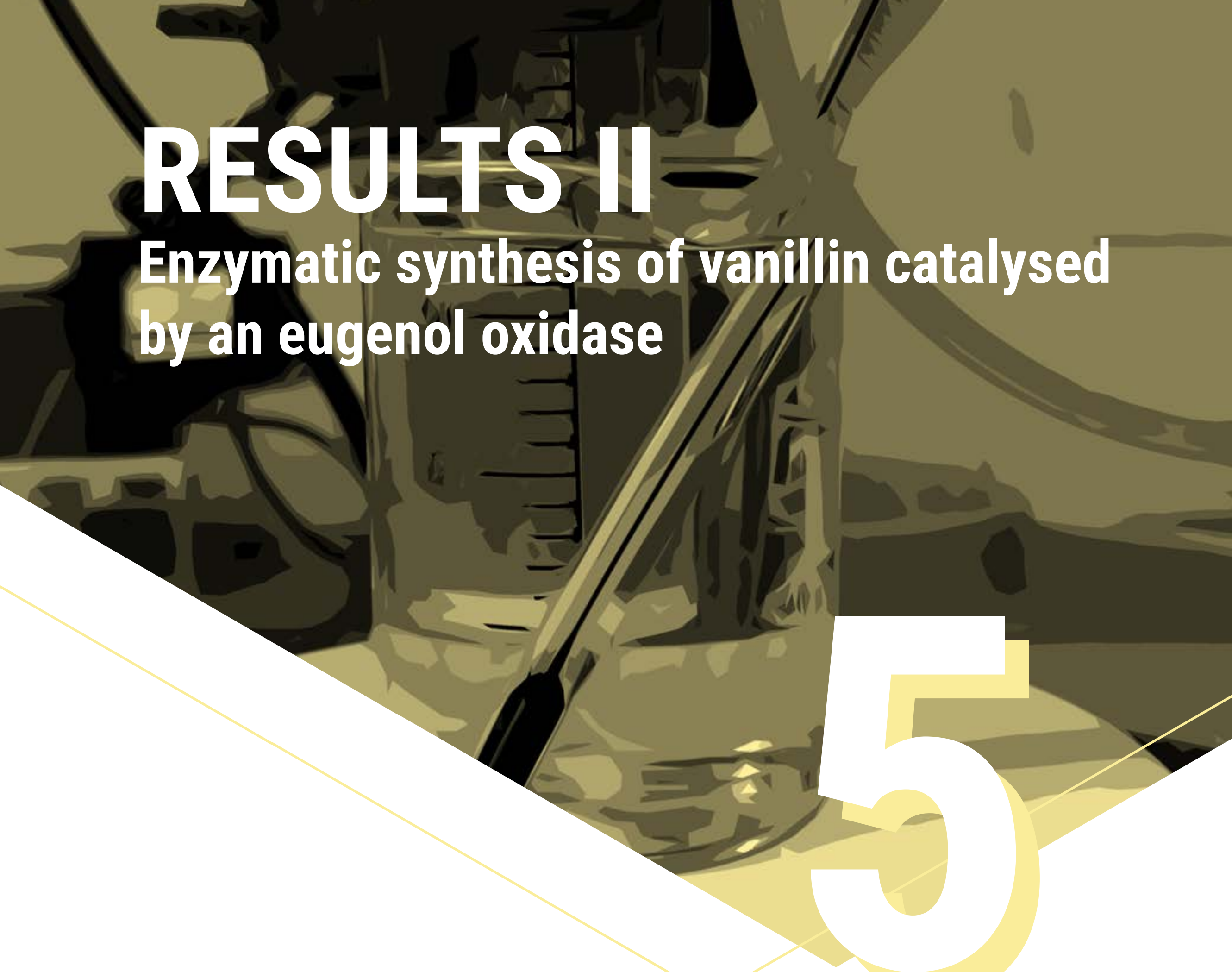


RESULTS II

Enzymatic synthesis of vanillin catalysed by an eugenol oxidase

5

Abstract

Vanillin is one of the most important flavours produced in the world. Due to its increasing value and its demand, mainly in the food industry, several ways to obtain it at lower prices are under study. One of the routes, here reported, is based on the oxidation of vanillyl alcohol by Eugenol oxidase (EUGO), which has high potential to be used at industrial scale owing to the high space time yields that can be obtained at lab scale (2.9 g L^{-1} h^{-1} of vanillin). Additionally, EUGO can be immobilised efficiently onto different supports (amino-agarose, epoxy-agarose-UAB M2 and Purolite® 8204F) which can be reused several times to perform the oxidation preserving good stability and improving more than 3-fold the biocatalyst yield, compared to reactions performed by the soluble EUGO.

5.1 Introduction

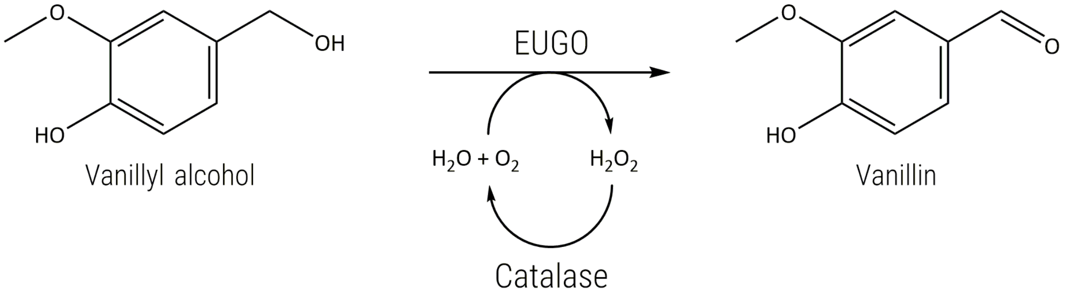
Natural vanilla (*Vanilla planifolia*; *V. pompona*) is the most important spice in the world, after saffron [91], with a current volume of almost 8 thousand tonnes per annum. 77 % of crop production is concentred in Madagascar, Indonesia and China and its production price over the last 10 years is around 5700 USD T^{-1} [130]. There are around 180 aromatic compounds in vanilla, and, the one that gives its characteristic flavour is vanillin (4-hydroxy-3-methoxybenzaldehyde) which only represents 2 % (w w^{-1}) of vanilla [131]. The difficulties of vanilla crop production, unpredictable climatic conditions, its rising high price (as high as silver) and its increasing demand, have all resulted in the development of synthetic methods of production of its flavouring compound, vanillin [132], [133]. It is estimated that less than 1 % of total world production of vanillin now comes from the natural beans [133]. The majority of vanillin production is performed by chemical synthesis. However, it is declining in interest in many markets (chiefly in the food industry) in favour of product produced by microorganisms, plants cells and enzymes, that can be labelled as "natural" [134]. Furthermore, natural vanillin can be sold at higher prices than the chemically synthesised product (1200 and 67.7 € kg^{-1}, respectively (Merck)), since consumers are increasingly willing to pay a premium for products produced by natural means [72], [135].

The high number of scientific papers and reviews published about vanillin bio-production by different pathways (enzymatic [92], [96],[136], microbial fermentation [137]–[145], plant cells cultures [146]), with different substrates (ferulic acid, lignin, eugenol, glucose, curcumin, etc) demonstrates the importance of natural vanillin and the need to obtain it in profitable ways by improving the metrics, enhancing the atom economy and reducing the costs, for its future industrial implementation [147]–[153].

Biotransformation processes using isolated enzymes can often be more expensive than those using whole cells due to the extra processing required during biocatalyst production, but this can be considerably offset by the reduction in secondary reactions, such as vanillic acid or vanillyl alcohol of the microbial production of vanillin, and reduced product purification costs.

One of the ways to improve the industrial viability of a bioprocess is to reuse the biocatalyst by immobilisation. Immobilisation does not only permit the reuse and easier recovery of the enzyme, but also allows the utilisation of different reactor configurations such as continuous or cross flow in addition to traditional batch formats and reduction of foam formation and can allow simplified product isolation [35], [49]–[51]. It can also improve biocatalyst stability towards various reaction parameters such as pH, temperature or organic solvent presence. Immobilisation can sometimes also enhance biocatalyst activity, specificity and selectivity, [49],[35],[50].

In the present chapter, Eugenol oxidase (EUGO) was immobilised covalently onto different supports and used to perform the conversion of vanillyl alcohol to vanillin (**Scheme 5.1**). The selected substrate is a by-product obtained during vanillin synthesis by fermentation from natural sources such as ferulic acid [94], [149]. The biocatalyst was reused, in order to improve the process metrics compared to reaction using soluble enzyme.



Scheme 5.1: Oxidation of vanillyl alcohol to vanillin catalysed by Eugenol oxidase (EUGO) and catalase.

5.2 Results and discussion

5.2.1 Characterisation of EUGO

Cell free extracts (CFE) containing EUGO activity were provided by InnoSyn B.V. These CFE were characterised regarding protein content (42.9 mg mL^{-1} CFE) and enzymatic activity (180.0 U mL^{-1} CFE). Aiming to choose the most suitable and appropriate conditions and supports for the immobilisation of EUGO, the effect of pH on stability was studied (**Figure 5.1**). EUGO was found to be stable over a wide pH range, preserving more than 90 % of its initial activity after 24 hours between pH 5.0 and 8.0 (**Figure 5.1**). However, at pH's higher than 8.0, the stability decreases rapidly. These results are well aligned with a previous publication by Q. Nguyen, *et al.* [95].

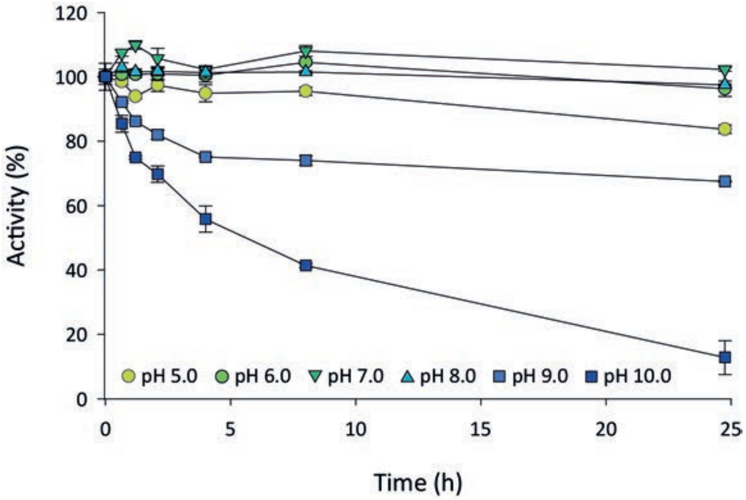


Figure 5.1: Influence of pH on EUGO stability. Stability was carried out by incubating 1 U mL^{-1} EUGO in 100 mM buffers: sodium acetate, pH 5.0; potassium phosphate, pH 6.0-8.0; Tris-HCl, 9.0, and carbonate-bicarbonate, pH 10.0, 25 °C.

5.2.2 Effect of hydrogen peroxide onto EUGO stability

Oxidations catalysed by EUGO produce H_2O_2 (**Scheme 5.1**) in stoichiometric amounts which could negatively affect the stability of the enzyme [154]. Therefore, the stability of EUGO was studied under reaction conditions at different concentrations of hydrogen peroxide (0-400mM) to analyse the effect of H_2O_2 on EUGO performance. The rank of concentrations was selected taking into account that the initial concentration of substrate used in the target reactions is set at 400 mM.

The obtained results are depicted in **Figure 5.2**, **A**. At 10 mM concentration, H_2O_2 had no impact on EUGO stability, whereas, at successively higher concentrations an increasingly negative impact was observed. After 24 hours, less than 50 % of initial activity was detected at concentrations higher than 50 mM. Therefore, due to the strong effect of H_2O_2 on EUGO stability, catalase was used in preparative reactions to convert it to water in-situ. Stability studies in the presence of up to 400 mM of H_2O_2 and catalase (9 mg mL^{-1} (35847 U mL^{-1})) showed no loss in EUGO activity compared to background (**Figure 5.2**, **B**). Therefore, it was decided to perform the oxidation of vanillyl alcohol in the presence of catalase.

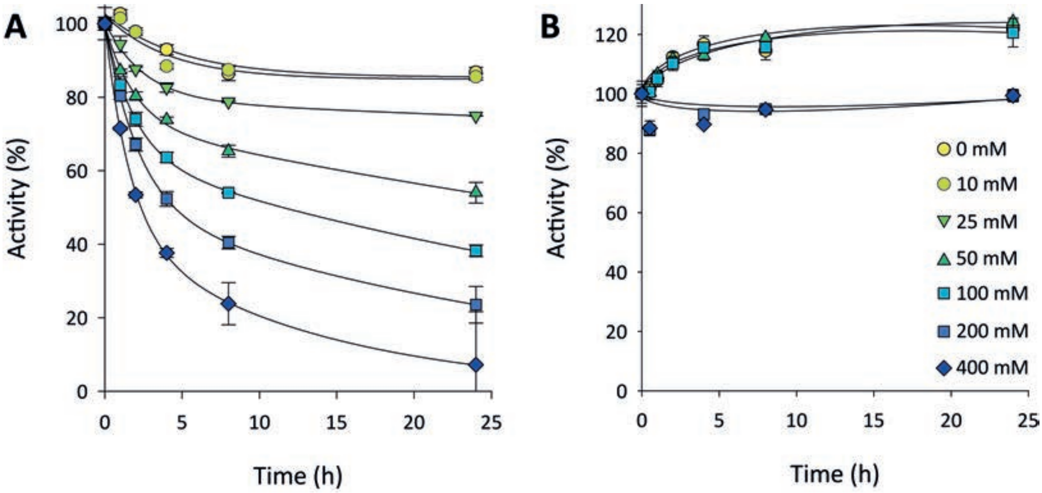


Figure 5.2: Stability of EUGO in reaction media (30% acetone, 50 mM potassium phosphate pH 7.5, mild agitation conditions, 25 ºC) at different concentrations of H_2O_2 of 0-400 (**A**) and with different concentrations of H_2O_2 (0-400 mM) and catalase (9 mg mL^{-1} (35847 U mL^{-1})) (**B**).

5.2.3 EUGO immobilisation

The extensive pH range where EUGO is stable allows the use of different immobilisation methods to attach the enzyme covalently onto different supports. Different supports with different matrices (agarose and methacrylate) and different functional groups (amino and epoxy) were selected for the immobilisation screening (**Table 5.1**).

Firstly, in order to ensure that the loading of enzyme offered to the supports did not show diffusional limitations, different enzyme loadings of 30, 10, 5 and 2.5 U mL^{-1} support were tested. Loadings of 10 U mL^{-1} support led to the retained activities showed in **Table 5.1**. When 5 U mL^{-1} was used the retained activities obtained were similar: 18 ± 2 % (epoxy-agarose-UAB M1; pH 7.5), 35 ± 4 % (epoxy-agarose-UAB M1; pH 8.0), 38 ± 3 % (epoxy-agarose-UAB M2; pH 8.0), 29 ± 5 % (Praesto epoxy 45), 44 ± 1% (Purolite® 8204F), 65 ± 5 % (amino-agarose), 35 ± 2 % (Amino ECR8409) and 21 ± 3 % (Amino ECR8415). Similar values were obtained when 2.5 U mL^{-1} was immobilised. When 30 U mL^{-1} was immobilised, retained activity values were lower, showing diffusional limitations. Therefore, loadings equal or lower to 10 U mL^{-1} did not show diffusional limitations for all the supports studied. Then, 10 U mL^{-1} support was the loading used in all the experiments to characterise the immobilisations.

Supports functionalised with amino groups (rows 6, 7, 8) behave as expected for ionic exchange resins. The enzyme initially becomes attached by ionic adsorption and in order to provide covalent linkages between carboxyl groups of the enzyme and amino groups of the support, CDI is used. The best result from this support type was obtained using amino-agarose, with 100 % immobilisation yield and 63 % retained activity.

Epoxy groups from supports react mainly with amino groups from the enzyme, but also with hydroxyl and thiol groups [119]. Alkaline pH favours this reaction because non protonated NH_2-groups are the reactive group. Firstly, the effect of pH on immobilisation was tested with epoxy-agarose-UAB M1. Two pHs (7.5 and 8.0 (rows 1 and 2)) were selected taking into account the stability decrease suffered by the EUGO at higher pHs (**Figure 5.1**). The best results were obtained at pH 8.0, as expected, reaching 97 ± 1 % and 37 ± 0 % immobilisation yield and retained activity respectively. These results represent an increase of 1.5-fold and 2.3-fold, respectively, compared to the experiment performed at pH 7.5.

Regarding the functional group density of the support, 9, 30 and 80 µmols of epoxy groups g^{-1} support were tested (row 4, 2 and 3) and the results show that increasing the activation grade from 9 to 30 µmols of epoxy groups g^{-1} support increase the immobilisation yield from 51 ± 1 % to 97 ± 1 % (rows 4 and 2). However, an increase from 30 to 80 did not significantly improve immobilisation yield or retained activity (rows 2 and 3). It was also noticed that using a support with similar activation grade but a different matrix did not lead to a variation of the immobilisation performance (rows 3 and 5).

Taking into account all the immobilisation studies, the best results were obtained with amino-agarose, epoxy-agarose-UAB M2 and Purolite® 8204F. They showed 100 ± 0 % immobilisation yield and 63 ± 2 %, 38 ± 1 % and 43 ± 0 % retained activity respectively.

These three supports, in bold in Table 5.1, were pre-selected to perform further experiments, in order to select the best immobilised derivative to perform the vanillyl alcohol oxidation reaction.

Table 5.1: EUGO immobilisation screening.

#	Support	Support activation grade (µmols g^{-1} support)	U of EUGO offered mL^{-1} support	Matrix	Support functional group	Immobilisation pH	Immobilisation yield (%)	Retained activity (%)
1	Epoxy-agarose-UAB M1	≈ 30	10	agarose	epoxy	7.5	64 ± 1	16 ± 1
2	Epoxy-agarose-UAB M1	≈ 30	10	agarose	epoxy	8.0	97 ± 1	37 ± 0
3	**Epoxy-agarose-UAB M2**	**≈ 80**	**10**	**agarose**	**epoxy**	**8.0**	**100 ± 0**	**38 ± 1**
4	Praesto epoxy 45 (Purolite®)	≈ 9	10	agarose	epoxy	8.0	51 ± 1	35 ± 1
5	**Purolite® 8204F (Purolite®)**	**≈ 73**	**10**	**Methacrylate**	**epoxy**	**8.0**	**100 ± 0**	**43 ± 0**
6	**Amino-agarose (ABT)**	**40-60**	**10**	**agarose**	**amino**	**6.0**	**100 ± 0**	**63 ± 2**
7	Amino ECR8409 (Purolite®)	unknown	10	Methacrylate	amino	6.0	100 ± 0	33 ± 1
8	Amino ECR8415 (Purolite®)	unknown	10	Methacrylate	amino	6.0	100 ± 0	22 ± 1

5.2.4 Stability of soluble and immobilised EUGO under reaction and storage conditions

The selected biocatalysts were incubated in reaction media (30 % acetone, 50 mM potassium phosphate pH 7.5, mild agitation conditions, 25 ºC) in order to analyse if immobilisation improved enzyme stability under these conditions compared to the soluble enzyme. Stability under storage conditions was also studied (25 mM potassium phosphate pH 6.0, 4 ºC). Under both conditions tested, soluble EUGO was found to be very stable, with a half-life of about 11.5 days under simulated reaction conditions. Similar profiles were observed for EUGO immobilised on amino-agarose ($t_{1/2}$: 8.3 days) and Purolite® 8204F ($t_{1/2}$: 17.3 days). In contrast, EUGO immobilised on epoxy-agarose-UAB M2 showed a 6-fold improvement, with a half-life of 77.5 days (Figure 5.3, **A**). Under storage conditions (Figure 5.3, **B**), both soluble and immobilised forms preserved more than 70 % initial activity after 14 days of storage.

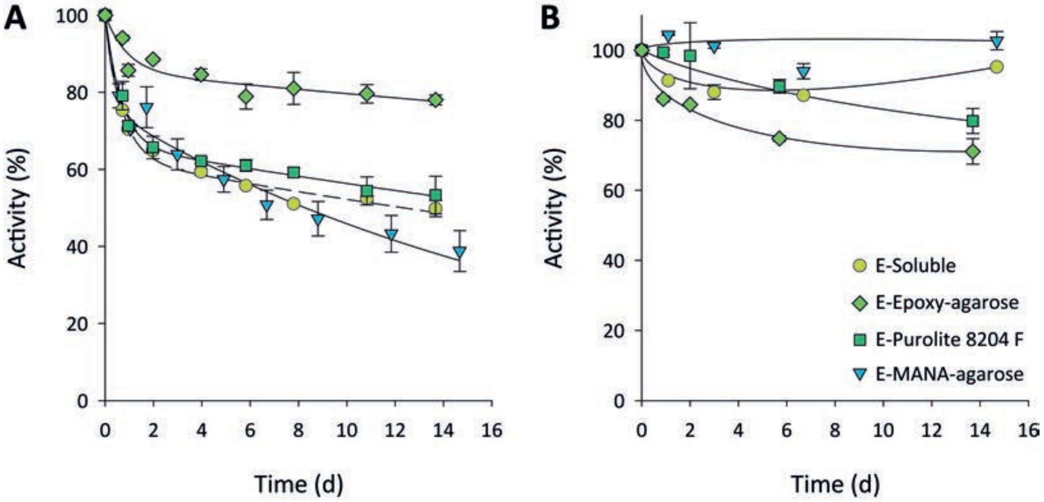


Figure 5.3: Stability of EUGO, soluble (○) and immobilised onto amino-agarose, epoxy-agarose-UAB M2 and Purolite® 8204F in reaction media (30% acetone, 50 mM Potassium phosphate pH 7.5, 25 ºC) (**A**) and in storage conditions (25 mM potassium phosphate pH 6.0, 4 ºC) (**B**).

5.2.5 Maximum loading of enzyme that can be immobilised onto the supports

The maximum quantity of enzyme that can be attached to each selected support was analysed in order to optimise the concentration of enzyme offered to the support without losing unnecessary amounts of biocatalyst in the supernatant. Different amounts of enzyme were immobilised onto the selected supports in order to determine this value. Results shown in Table 5.2 determined that EUGO can be immobilised efficiently at high loadings onto amino-agarose, epoxy-agarose-UAB M2 and Purolite® 8204F. Amino-agarose and epoxy-agarose-UAB M2 can be loaded up to 456-509 U mL^{-1} support, without high enzyme loss in the

Table 5.2: Results of maximum enzyme loading immobilised on the selected supports, following the procedures described in sections 3.3.5.1, 3.3.5.2 and 3.3.7. Experiments were performed by duplicate.

Support	**U offered mL^{-1} support**	**% immobilisation**	**theorical U immobilised mL^{-1} support**	**Theoretical retained activity (U mL^{-1} support)**	**Measured activity of the immobilised derivative (U mL^{-1})**
Amino-agarose (RA: 63%)	152	99 ± 0	151 ± 0	96 ± 0	35 ± 1
	304	99 ± 0	302 ± 0	191 ± 0	40 ± 3
	456	**97 ± 0**	**442 ± 0**	**279 ± 0**	**39 ± 2**
	608	81 ± 1	491 ± 4	310 ± 2	39 ± 7
	759	64 ± 2	487 ± 18	308 ± 12	34 ± 16
Epoxy-agarose-UAB M2 (RA: 38%)	170	99 ± 0	169 ± 0	63 ± 0	29 ± 3
	340	98 ± 0	334 ± 1	125 ± 0	31 ± 3
	509	**94 ± 1**	**478 ± 3**	**179 ± 2**	**32 ± 1**
	679	85 ± 0	577 ± 1	216 ± 0	32 ± 3
	849	76 ± 0	649 ± 1	244 ± 1	33 ± 0
Purolite® 8204F (RA: 43%)	170	98 ± 0	167 ± 1	72 ± 0	19 ± 3
	340	**88 ± 1**	**300 ± 3**	**131 ± 2**	**29 ± 9**
	509	71 ± 2	361 ± 11	157 ± 7	29 ± 7
	679	61 ± 0	414 ± 3	180 ± 2	22 ± 5
	849	54 ± 1	460 ± 6	200 ± 4	22 ± 2

* Theoretical retained activity in the absence of diffusional limitations

supernatant. That equates to 442 ± 0 and 479 ± 3 U immobilised mL^{-1} support respectively (in bold, Table 5.2). At high loadings of enzyme, the activity of immobilised derivatives cannot be analysed due to the presence of diffusional limitations. In order to determine the retained activity, it is supposed that the enzyme retains the same percentage of activity as determined during immobilisation at low enzyme loading (without diffusional limitations). Taking into account the retained activity percentages determined in the characterisation (i.e. 63 % for amino-agarose and 38 % for epoxy-agarose-UAB M2) the supports can retain up to 279 ± 0 and 179 ± 2 U mL^{-1} of support. Regarding the Purolite® 8204F, the methacrylate-based support could be loaded with up to 340 U mL^{-1} support preserving high immobilisation yield, which corresponds to a retained activity of 130 U mL^{-1} of support (in bold, Table 5.2).

As expected, measured activities of the immobilised derivatives at these loadings (39 ± 0, 32 ± 0 and 29 ± 0 U mL^{-1} support for amino-agarose, epoxy-agarose-UAB M2 and Purolite® 8204F) were lower than the theoretical values, probably due to diffusional limitations.

5.2.6 Vanillyl alcohol oxidation using soluble and immobilised EUGO

Preparative scale vanillin synthesis reactions were performed on a 10 mL scale with a high concentration of the substrate vanillyl alcohol (400 mM) according to the industrial requirements. In order to increase the solubility of the substrate it was needed the use of 30 % v v^{-1} acetone as a cosolvent, as shown in Section 3.3.9.1. 10 % v v^{-1} of immobilised enzyme on amino-agarose (279 U mL^{-1} of support), epoxy-agarose-UAB M2 (179 U mL^{-1} of support) and Purolite® 8204F (131 U mL^{-1} of support) were used. For comparative purposes the same overall number of units of immobilised enzyme to soluble enzyme was used to compare process metrics. All reactions also contained 35847 U of catalase mL^{-1} of reaction media to eliminate the peroxide formed by EUGO. Reactions with 28, 18 and 13 U mL^{-1} of soluble EUGO gave high conversions of 86, 86 and 84 % after 30, 41 and 37 hours respectively (Table 5.3).

Reactions with immobilised EUGO gave comparable conversions to that of the free enzyme when using similar overall number of units activity under similar reaction conditions.

Similar space-time yields were obtained with both forms of epoxy-agarose-UAB M2 (immobilised and soluble) (1.8 and 1.6 g P L^{-1} h^{-1}, respectively) and Purolite® 8204F (1.2 and 1.4 g P L^{-1} h^{-1}, respectively), but a 50 % increase was observed with EUGO immobilised on amino-agarose (2.9 g P L^{-1} h^{-1}). This variation was produced because the reaction time was lower in the reaction with immobilised derivative. This behaviour might occur because the retained activity of the immobilisation at high loadings, which we assume that is similar to the immobilisation at low loadings (without diffusional limitations), in this case is higher, thus a higher activity would have been offered to the reaction. Soluble EUGO gave a space-time yield of 1.9 g P L^{-1} h^{-1}. These high values, compared with other biotechnological ways to produce vanillin [136], [137], [139], [144], [145], [156], make EUGO a promising enzyme to perform the vanillyl alcohol oxidation at industrial scale.

Regarding hydrogen peroxide, the presence of catalase avoided the deactivation of the enzyme. However, the high k_M of catalase for hydrogen peroxide (33.3 mM) [157], resulted in a certain presence of hydrogen peroxide which can react with vanillin triggering the Dakin oxidation reducing the reaction yield [158]. This oxidation produced a dark colour according to InnoSyn data (Unpublished results).

5.2.7 Reusability of immobilised EUGO for vanillyl alcohol oxidation

Immobilised derivatives were each reused over 5 reaction cycles and the results are shown in Table 5.3 and Figure 5.4. Purolite® 8204F displayed lowest recyclability, retaining less than 65 % conversion and less than 15 % yield by the last cycle. EUGO immobilised onto epoxy-agarose-UAB M2 and amino-agarose showed better operational stability with conversions higher than 80 %, for both derivatives and yields higher than 50 % and 30 % in the last cycle, respectively. The biocatalyst yield improved more than 2-fold compared to soluble enzyme, with a maximum obtained using the epoxy-agarose-UAB M2 immobilised derivative (53.6 mg P mg^{-1} EUGO). It should be mentioned that the particles were abraded by magnetic stirring during the re-cycles and, therefore, the particle size was reduced during the experiments. This effect can produce a decrease in diffusional limitations which would increase the

Table 5.3: Process metrics obtained in the vanillyl oxidation using soluble and immobilised EUGO. 400 mM of vanillyl alcohol, 30% (v v^{-1}) acetone in 50 mM potassium phosphate buffer at pH 7.5, 25 ºC, 1 vvm air (hydrated with a 30% acetone solution), magnetic stirring (500 rpm), and 10 µL antifoam. 9 mg mL^{-1} reaction (35847 U mL^{-1} reaction) of catalase.

Biocatalyst	Form	U mL^{-1} reaction	Reaction time (h)	Conversion (%)	Total vanillin in solution (g)	Biocatalyst* yield (mg P mg^{-1} EUGO offered)
Amino-agarose	Soluble	28	30	86	0.4	10.6
	Immobilised		18	87**	1.1	29.8
Epoxy-agarose-UAB M2	Soluble	18	41	86	0.4	16.6
	Immobilised		30	90**	1.3	53.6
Purolite® 8204F	Soluble	13	37	84	0.3	19.5
	Immobilised		36	76**	0.7	41.2

*Determined when the reaction finished.

**Overall conversion.

initial reaction rate. However, this effect would be counteracted by the activity decrease due to reduced enzyme stability. The reduction of the particles also triggered filtering difficulties by obstruction of the reactor funnel filter which prolonged the immobilised derivative washing steps. This effect can be avoided by using a higher reactor with mechanic stirring instated of magnetic stirring. As the reactions performed with soluble EUGO, there was a high difference between conversion and yields, which could be caused by the Dakin oxidation (explained above) and by stripping losses of product and/or substrate.

Only one study was found in the literature that reported vanillin production with an immobilised enzyme (oxygenase Cso2) [136]. Although a different pathway (from isoeugenol) was used, they obtained 0.68 mg of vanillin per mL over ten cycles. We could produce 45 mg of vanillin mL^{-1} in 5 cycles when amino-agarose was used as immobilisation carrier, which represents an increase of 66-fold over the alternative literature procedure.

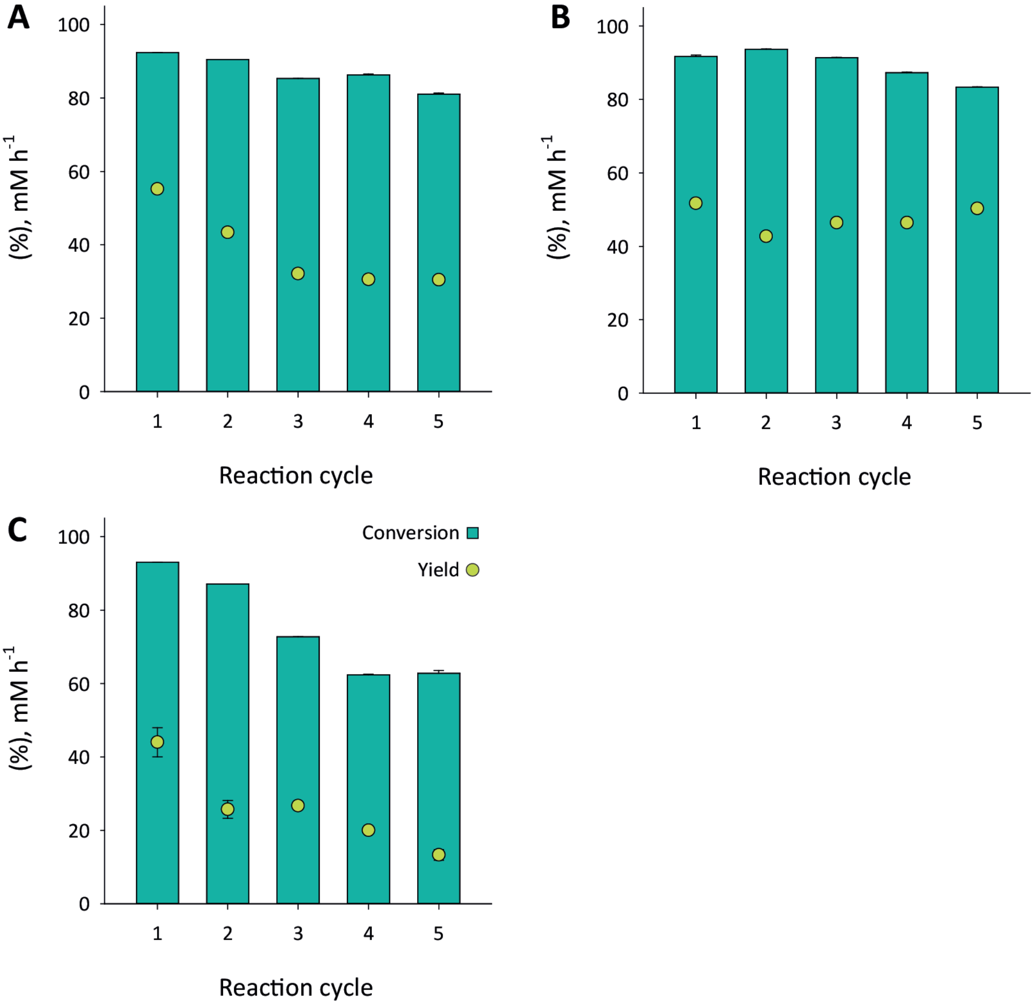
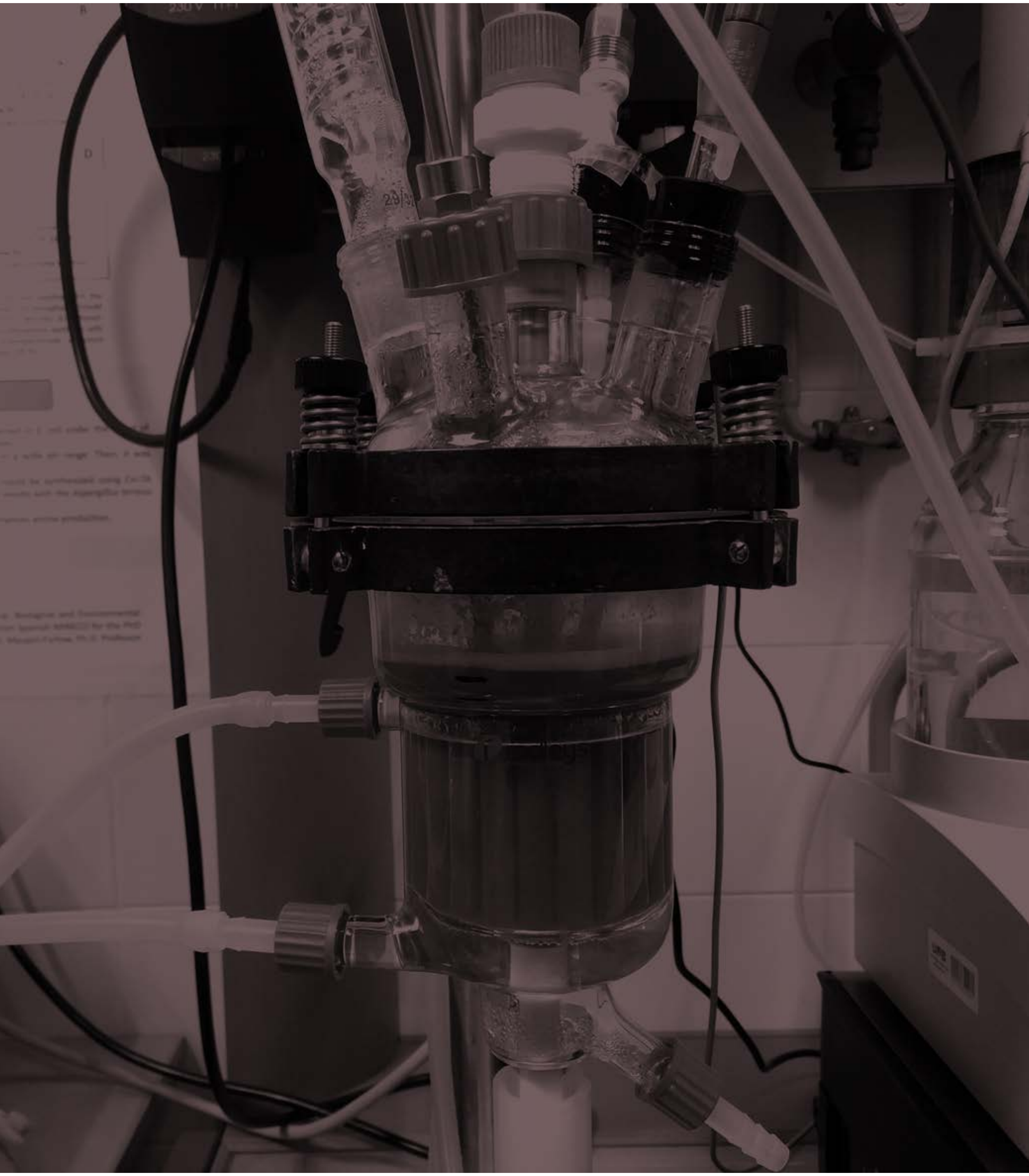
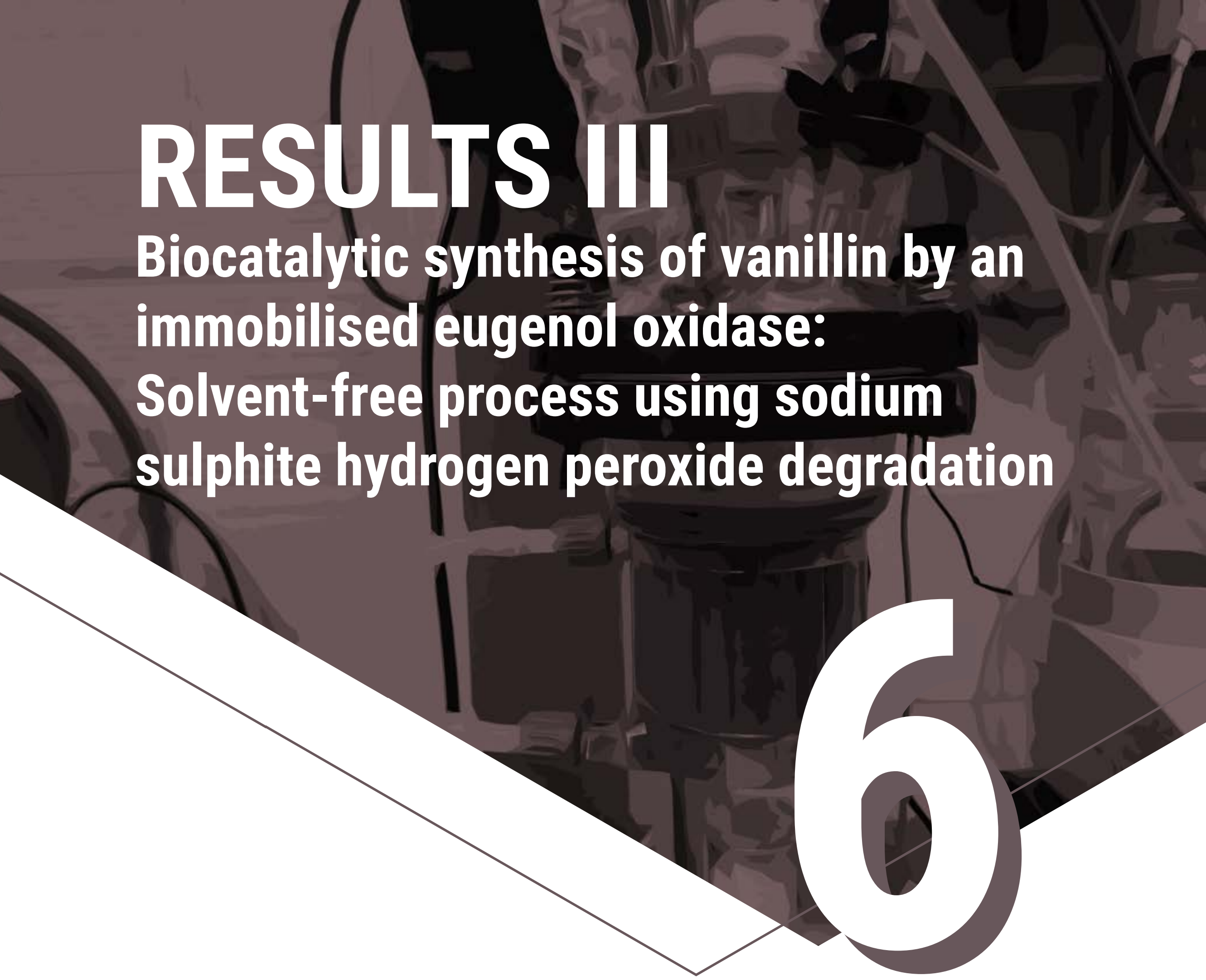


Figure 5.4: Conversion and Yield of the reaction cycles with amino-agarose 283.5 U mL^{-1} support (**A**), epoxy-agarose-UAB M2 182.8 U mL^{-1} support (**B**) and Purolite® 8204F 124 U mL^{-1} support (**C**), and Catalase (35847 U mL^{-1} reaction). Reaction conditions: 10 mL, 25 °C, magnetic stirring (500 rpm), 1 vvm, 400 mM Vanillyl alcohol/50 mM Potassium phosphate pH 7.5.

5.3 Conclusions

Eugenol oxidase is an active and stable enzyme over a wide range of pH. It can be immobilised efficiently into different supports (amino-agarose, epoxy-agarose-UAB M2 and Purolite® 8204F) at high enzyme loadings (279, 179 and 131 U EUGO mL^{-1} support, respectively). Immobilised derivatives showed high stability under the reaction conditions used with a 6-fold improvement in half-life for EUGO immobilised onto epoxy-agarose-UAB M2 compared with the soluble form. High conversions (more than 90 %) and space-time yield of 2.9 g vanillin L^{-1} h^{-1} could be obtained in the vanillyl alcohol oxidation with soluble and immobilised forms. The immobilised derivatives could be reused satisfactorily for more than 5 cycles, with EUGO immobilised into epoxy-agarose-UAB M2 being the operationally most stable biocatalyst tested, retaining more than 80 % of conversion in the last cycle, which represents 90 % of retained activity compared to fresh biocatalyst. It presented the highest biocatalyst yield of 53.6 mg vanillin mg^{-1} EUGO which is 3-fold more than the soluble form. Changes in operational parameters as use of mechanic stirring and a more efficient method for the elimination of hydrogen peroxide to avoid product loss can trigger into process improvements. The results of this chapter have been published [101].





RESULTS III

Biocatalytic synthesis of vanillin by an immobilised eugenol oxidase: Solvent-free process using sodium sulphite hydrogen peroxide degradation

6

Abstract

Vanilla is the second most expensive spice in the world. This issue triggered the necessity to obtain its flavour compound, vanillin, by alternative methods, most of which have focused on biosynthesis since it can be labelled as “natural” and sold at higher price. In this work, a process for the enzymatic synthesis of vanillin has been optimised, not only from a process metrics point of view but also from an environmentally sustainable perspective. The maximum biocatalyst activity and stability was taken into account for the selection of the reaction conditions. Soluble and immobilised eugenol oxidase (EUGO) was tested under both reaction conditions. The optimum conditions obtained lead to an organic solvent free process reaching 9.9 g P L^{-1} h^{-1}. Moreover, the use of immobilised EUGO allowed the biocatalyst to be reused up to 18 reaction cycles, improving the biocatalyst yield more than 12-fold, thus also reducing the biocatalyst associated cost.

6.1 Introduction

In the present chapter, vanillin synthesis from vanillyl alcohol using soluble and the selected EUGO immobilised derivative in the previous chapter (EUGO-epoxy-agarose-UAB M2) (**Scheme 5.1**) has been studied and optimised by reaction medium engineering in order to bring the process closer to industrial implementation. Different pH conditions have been compared: pH conditions for maximum enzymatic stability [101] and, as alternative, an optimised reaction conditions by Straatman *et al.* [102] from InnoSyn as part of ROBOX project. These new conditions use a higher pH which leads to the maximum enzyme activity also increasing substrate solubility, thus circumventing the use of organic solvent in the media. Moreover, the use of Na_2SO_3 was also considered to improve H_2O_2 removal as compared to catalase use which can produce side reactions (Dakin reaction) and can affect the EUGO stability [101].

Additionally, the reaction conditions described in the previous chapter are not recommended to be applied at industrial scale from a safety point of view, since acetone can react with hydrogen peroxide producing an explosive (triacetone triperoxide). In the reaction conditions proposed in this chapter acetone was avoided.

Reaction medium engineering has, therefore, been carried out aiming not only to improve process metrics but also to develop a greener and industrially implementable process avoiding the use of organic solvent.

6.2 Results and discussion

6.2.1 Effect of pH on EUGO activity

EUGO activity in relation to the reaction pH was studied in the present work (**Figure 6.1**). EUGO reached the optimum reaction rate around pH 9.5. The enzyme showed a strong activity dependence towards pH, decreasing to almost 50 % at pH values higher than 10.0 and lower than 8.5. The stability of EUGO towards pH has been previously reported, with highest stability between pH 6.0 and pH 8.0 [101]. Both pH values, the one for maximum activity (pH 9.5) and the one for optimum stability (pH 7.5), were tested in the reaction in order to determine which conditions lead to higher process metrics such as product concentration, total product and biocatalyst yield.

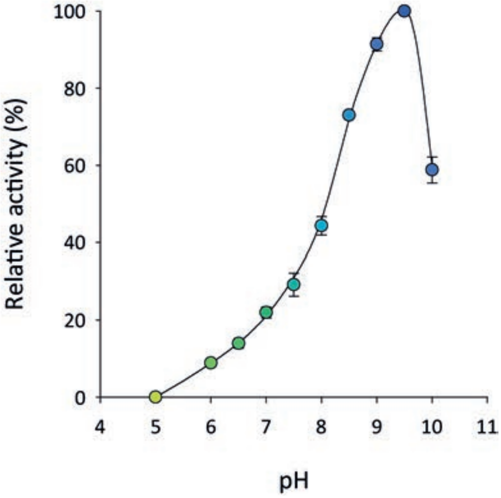


Figure 6.1: Effect of pH on EUGO activity. Activity was measured at pH 5.0-10.0. Substrate solution was prepared in the following 50 mM buffers: sodium acetate (pH 5.0), potassium phosphate (pH 6.0-8.0) and Glycine-NaOH (pH 8.5-10.0), 30 ºC.

6.2.2 Vanillyl alcohol oxidation using soluble EUGO under optimum stability pH (conditions A) and optimum activity pH (conditions B)

EUGO displays maximum activity at pH 9.5 (**Figure 6.1**), although maximum stability is observed at pH 7.5 (**Figure 5.1**) [101]. Both reaction conditions, best stability (pH 7.5; **con-**

ditions A) and best activity (pH 9.5; **conditions B**) were compared (see Section 3.3.9.3). Using conditions A, an 86 % conversion and 70 % solution yield was obtained, with a Space-Time Yield (STY) of 1.5 g P L^{-1} h^{-1} and 16.6 mg P mg^{-1} EUGO biocatalyst yield after 28 h of reaction (Figure 6.2, **A**, Table 6.1). These conditions required the use of acetone as a cosolvent to dissolve the substrate (400 mM). At higher pH (conditions B) vanillyl alcohol is more soluble, so acetone addition to enhance solubility is not required. The use of acetone in the process can therefore be avoided, making the process i) greener by circumventing the environmental issues associated with volatile organic solvents and ii) less hazardous, as H_2O_2 is no longer present with acetone.

Considering the high K_M of catalase (33.3 mM) [157], hydrogen peroxide may be present under reaction conditions as evidenced by side reactions, albeit at a concentration that does not affect EUGO activity [101]. To remove the produced H_2O_2 in a more efficient way, Na_2SO_3 was instead added to the reaction (conditions B), which reacts with hydrogen peroxide, producing water and avoiding side reactions [159].

Reactions performed under the new conditions, where EUGO has maximum activity (conditions B), reduced the reaction time 7.1-fold when compared with the original reaction

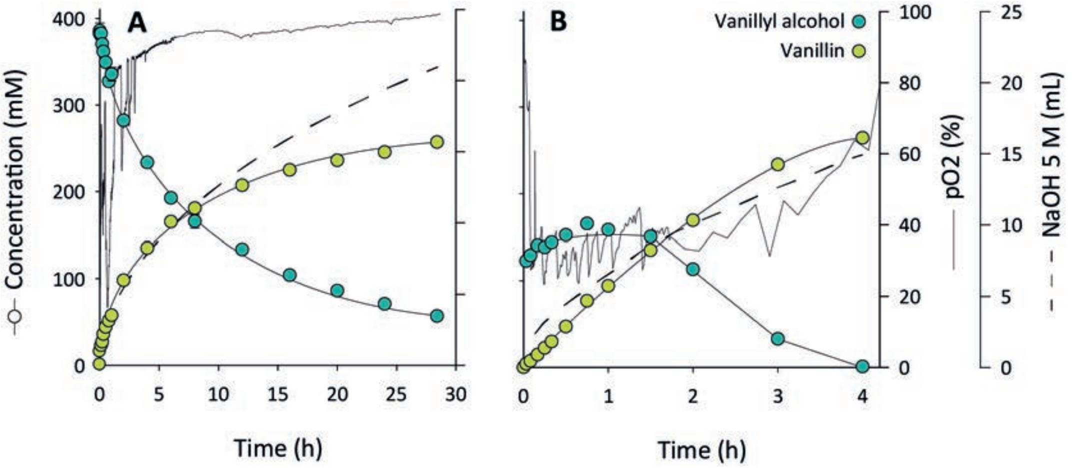


Figure 6.2: Substrate, Product, NaOH and pO_2 profiles. Reaction conditions: soluble EUGO (18 U mL^{-1} reaction) 250 mL reaction volume, 25 °C, 1000 rpm, 1 vvm air with 30% acetone in 50 mM potassium phosphate pH 7.5 with 400 mM vanillyl alcohol and catalase (35847 U mL^{-1} reaction), 1 M NaOH titration (**A**) or 50 mM glycine-NaOH pH 9.5 with 330 mM vanillyl alcohol, 440 mM Na_2SO_3, 5 M NaOH titration (**B**).

conditions performed under conditions giving maximum EUGO stability (conditions A), obtaining complete conversion and 88 % solution yield after just 4 h (**Figure 6.2**, **B**, **Table 6.1**). This resulted in a 6.5-fold increase in STY to 9.9 g P L^{-1} h^{-1}. Although the reaction had 17.5 % less substrate, biocatalyst yield and total production were slightly higher (1.1-fold) than obtained previously (18.2 mg P mg^{-1} EUGO and 11.2 g P, respectively). These increases were obtained due to the higher rate of conversion and the decrease in by-product formation, probably due to the beneficial effect of high pH on enzyme activity and more efficient H_2O_2 removal when using Na_2SO_3 instead of catalase. These results fit with the appearance of both reactions, where, as mentioned in the previous chapter, a dark colour was produced under conditions A (Dakin reaction, according to InnoSyn data (Unpublished results)) that was not observed under conditions B. It should be noted that substrate concentration increases during the first 1 h of the process as the substrate it not fully dissolved at the beginning of the reaction under the new solvent-free conditions. Therefore, the vanillyl alcohol concentration measured during this period is a combination of both substrate dissolution and substrate consumption (**Figure 6.2**, B).

These results were very promising compared with others reported in the literature. The majority of recent papers published about vanillin are focused on the fermentation of cheap raw materials, most of them with recombinant microorganisms via *de novo* synthesis in order to obtain higher productivities. However, public opinion about the use of genetic tools to produce food compounds is still reticent [153]. Nonetheless, the majority of productivities reported are still lower than 0.5 g L^{-1} h^{-1} [160]–[167]. Moreover, it should be mentioned that no vanillic acid was detected applying the strategy described in the present work (since EUGO, under these conditions, does not oxidise the aldehyde to an acid), unlike most of vanillin production processes based on microbial fermentation. Microorganisms do not accumulate high concentrations of aldehydes as vanillin, due to their chemical reactivity and also may be toxic for the cells. Therefore, microorganisms tend to convert vanillin to less toxic compounds, such as vanillyl alcohol or vanillic acid, which could be obtained at high concentrations [149], [166], [168]–[170].

Other authors have reported the production of vanillin by biotransformation.

Ni *et al.* [171] reported 1.1 g L^{-1} h^{-1} using a whole cell biocatalyst. Yamada *et al.* [172] reported a high STY of 4.7 g L^{-1} h^{-1} by whole cell biotransformation from isoeugenol using a recombinant *E. coli* expressing the isoeugenol monooxygenase gene of *Pseudomonas putida* IE27, obtaining a high molar conversion yield of 81 % after 6 h reaction, where presence of vanillic acid was detected. A similar procedure to the Yamada *et al.* [172] has also been patented recently by Bruhlmann, obtaining lower STY (about 1.2 g L^{-1} h^{-1}) [84]. Klaus *et al.* [173] produced vanillin by a one-pot, three-step enzymatic cascade using recombinant P450 and vanillyl alcohol oxidase (VAO) from a cheap substrate (3-methylanisole), but a low STY was obtained. 2.9 g L^{-1} h^{-1} of vanillin could be obtained from vanillyl alcohol by isolated EUGO in a previous work, where no vanillic acid was produced [101]. In the present work, changing the reaction conditions, to achieve the maximum activity, 9.9 g L^{-1} h^{-1} was obtained which represents almost double the STY obtained by the best results found in the literature [172].

6.2.3 Vanillyl alcohol oxidation by immobilised EUGO under both reaction conditions

The EUGO immobilised derivative selected in the previous chapter, EUGO-epoxy-agarose-UAB M2, which showed 100 % immobilisation yield, 38 % remaining activity and had a specific activity of 180 U mL^{-1} support, was used to perform the reactions with immobilised derivative of the present chapter.

Vanillin synthesis was performed from vanillyl alcohol catalysed by immobilised EUGO. The reactions were performed under the same conditions (A and B) which were used with soluble EUGO and also the same activity loadings in units of catalyst mL^{-1} of reaction volume (18 U mL^{-1} reaction volume).

The reactions behaved in a similar manner to those performed using soluble EUGO. Reactions performed using conditions B required 4.5 h to reach completion, where full conversion was obtained with a 90 % solution yield of the desired product. The reaction time was 5.3-fold lower than the reaction performed under conditions A, where an 84 % con-

Table 6.1: Process metrics obtained in the vanillyl alcohol oxidation using soluble and immobilised EUGO (18 U mL^{-1} reaction), 250 mL reaction volume, 25 ºC, 1000 rpm, 1 vvm air, under both conditions studied: 30% acetone in 50 mM potassium phosphate pH 7.5 with 400 mM vanillyl alcohol and catalase (35847 U mL^{-1} reaction), 1 M NaOH titration (A). 50 mM glycine-NaOH pH 9.5 with 330 mM vanillyl alcohol, 440 mM Na_2SO_3, 5 M NaOH titration (B).

Form	Conditions	Reaction time (h)	Total vanillin (g)	Conversion (%)	Solution Yield (%)	STY (g P L^{-1} h^{-1})	Biocatalyst yield (mg P mg^{-1} EUGO)
Soluble	A	28	10.2	86	70	1.5	16.6
Soluble	B	4.0	11.2	100	88	9.9	18.2
Immobilised	A	24.0	10.6	84	70	1.6	17.2
Immobilised	B	4.5	11.3	100	90	9.2	18.3

version and 70 % solution yield were achieved. Similar STY and biocatalyst yield (1.6 g P L^{-1} h^{-1}, 17.2 mg P mg^{-1} EUGO and 9.2 g P L^{-1} h^{-1}, 18.3 mg P mg^{-1} EUGO, for conditions A and B, respectively) were obtained, compared to reactions using soluble EUGO under both conditions (**Table 6.1**).

6.2.4 Reusability of EUGO immobilised derivatives

In order to test the reusability of immobilised EUGO. The immobilised derivative was repeatedly used during different cycles of vanillin synthesis under the mentioned reaction conditions (A and B).

Fresh immobilised derivatives were used in an initial reaction to reach complete conversion (24 and 4.5 h for conditions A and B respectively). Subsequently they were reused in new reactions run for the same time period as the initial reaction and cycling continued until conversions dropped to below 50 %. Immobilised EUGO biocatalysts could be recycled 5 and 18 times using reaction conditions A and B, respectively (**Figure 6.3**).

These recycles allowed 40 g and 138 g quantities of vanillin, calculated based on solution yields, to be produced from conditions A and B respectively. This represents a 3.5-fold increase in vanillin production compared with a single cycle using the same immobilised derivative. Recycling led to 65.0 mg P mg^{-1} EUGO and 225.1 mg P mg^{-1} EUGO for conditions A and B, respectively, improving the biocatalyst yield 3.9-fold and 12.4-fold respectively, compared to reactions with soluble enzyme under the same conditions.

These results are encouraging compared to those reported in the literature on vanillin production by immobilised enzymes. Furuya *et al.* [136] could perform 10 reaction cycles with oxygenase Cso2 producing 0.68 mg P mL^{-1}. In the present work the biocatalyst yield and the STY has been improved by 4.2-fold and 3.2-fold respectively compared to a previous work [101], corresponding to a 66-fold improvement with respect to the results reported by Furuya *et al.*

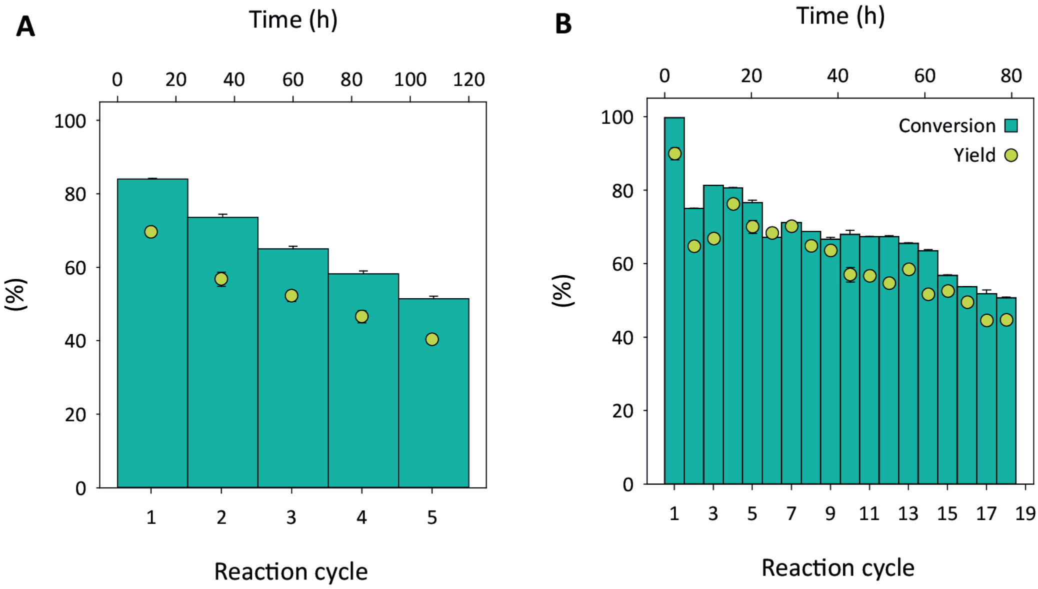


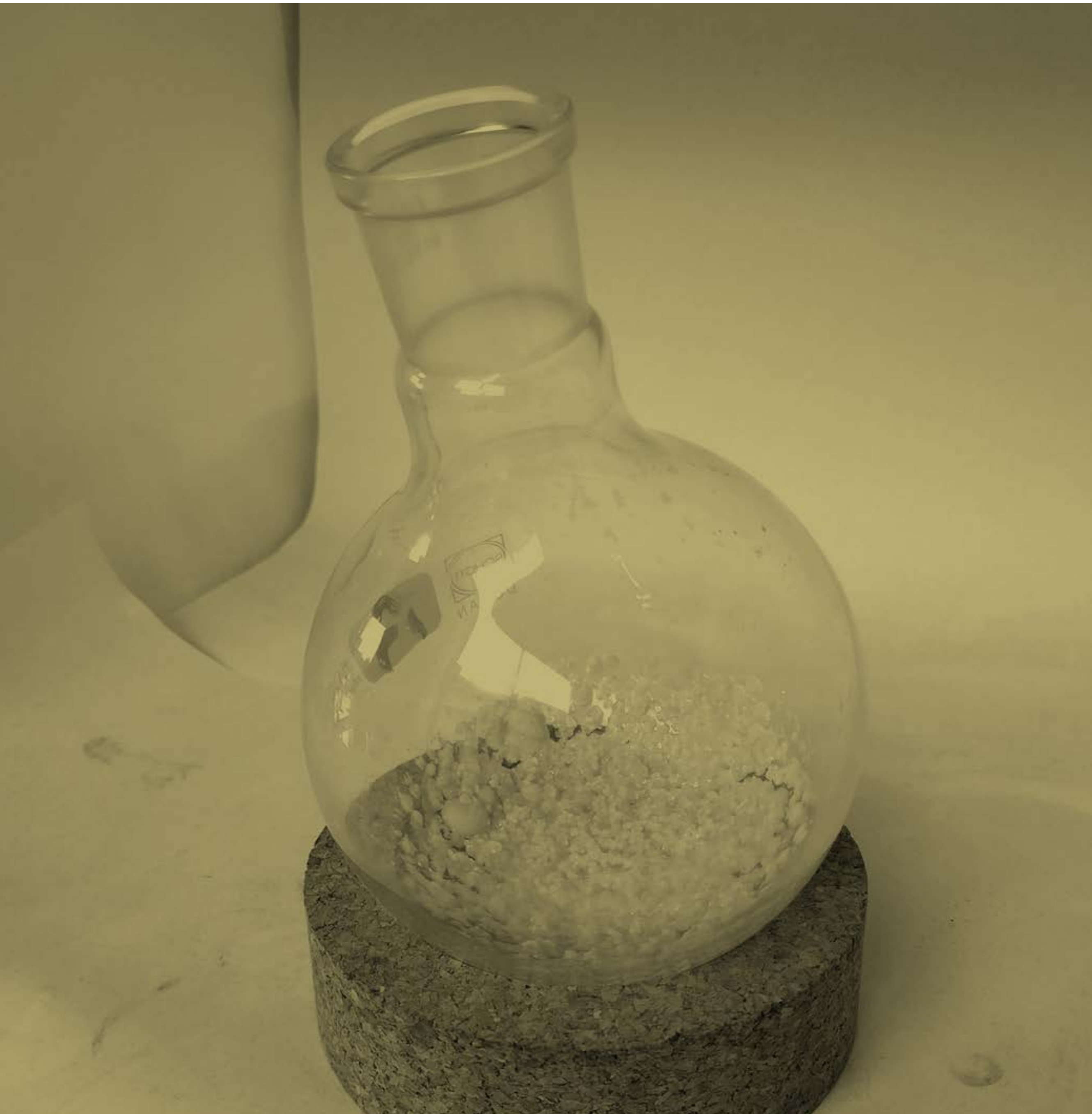
Figure 6.3: Conversion and solution Yield of the reaction cycles with EUGO-epoxy-agarose-UAB M2 (18 U mL^{-1} reaction), 250 mL reaction volume, 25 ºC, 1000 rpm, 1 vvm air. Reaction conditions: 30% acetone in 50 mM potassium phosphate pH 7.5 with 400 mM vanillyl alcohol and catalase (35847 U mL^{-1} reaction), 1 M NaOH titration (**A**). 50 mM glycine-NaOH pH 9.5 with 330 mM vanillyl alcohol, 440 mM Na_2SO_3, 5 M NaOH titration (**B**).

6.2.5 Vanillin isolation

Vanillin extraction, following the procedure described in Section 3.3.9.5 (250 mL reaction, conditions B), gave 10.34 g of vanillin in 85 % isolated yield determined by HPLC analysis. The product obtained, with a purity of 98 % (determined by HPLC), was identical to authentic vanillin by ^{1}H and ^{13}C NMR 600 MHz in $CDCl_3$ and literature data (Appendix Figure 8.6 and 8.7) [174].

6.3 Conclusions

Eugenol oxidase is a promising enzyme to produce “natural” vanillin at industrial scale. EUGO is stable and active over a wide pH range, but the maxima for each parameter do not overlap. For this reason, two conditions have been compared: maximum stability (pH 7.5, conditions A) and maximum activity (pH 9.5, conditions B). The increased activity under conditions B allowed a reduction in reaction time to 4 h when soluble EUGO was used, increasing the STY 6.5-fold (compared with conditions A) obtaining 9.9 g L^{-1} h^{-1}. When EUGO was immobilised onto epoxy-agarose-UAB M2, the same behaviour was observed as with soluble enzyme for both conditions. Immobilised derivatives could be reused up to 5 and 18 cycles, with conditions A and B respectively. Despite of the fact that the operational stability under conditions B was reduced compared to conditions A (81 vs. 120 h), this was more than off-set by the fast cycle time, allowing 138 g of vanillin to be produced using conditions B, increasing the biocatalyst yield to 225.1 mg P mg^{-1} EUGO which represents a 12.4-fold and 3.5-fold improvement compared with single use of the soluble enzyme and recycling immobilised enzyme using conditions A respectively. In addition, conditions B are greener and more industrially implementable, since acetone is avoided. Moreover, the biocatalyst associated cost is reduced due to an increase in biocatalyst yield achieved by enzyme recycling. These promising results, bring the possibility of using biosynthesis of vanillin closer to industrial implementation. The research of this chapter has been recently published [175].



OVERALL CONCLUSIONS

7

Use of ADH and AOX to convert alcohols in two valuable products

In this thesis, reaction engineering and immobilisation were used to improve the process metrics of two target reactions of industrial interest: i) the oxidation of chlorolactol to produce chorolactone catalysed by ADH99/NOX and ii) the oxidation of vanillyl alcohol to produce vanillin catalysed by EUGO, to bring the implementation of this biocatalytic processes closer to industrial implementation. The specific conclusions are:

- ADH99, NOX and EUGO were immobilised efficiently onto different supports retaining activities up to 62 %, 37 % and 63 %, respectively.
- The stability of ADH99 and EUGO immobilised derivatives could be enhanced, improving the half-life up to 13.7 and 6-fold, respectively. However, NOX stability was not improved when immobilised.
- All the enzymes could be immobilised onto the selected supports at high enzyme loadings, up to 523, 468 and 479 U mL^{-1} support for ADH99, NOX and EUGO on epoxy-agarose-UAB M2, amino-agarose and epoxy-agarose-UAB M2, respectively.
- ADH99-epoxy-agarose-UAB M2 was selected as the best immobilised derivative. In the case of EUGO, the 3 supports that showed better results were selected. NOX was selected for use in soluble form since immobilisation did not improve its stability.
- The reaction conditions (substrate concentration, reaction time and ratio of ADH99-NOX) of the chlorolactol oxidation were optimised using soluble enzymes obtaining 94.7 % yield, 4.6 g P L^{-1} h^{-1} of Space-Time Yield and 7.9 mg P mg^{-1} B of Biocatalyst yield.
- The ADH99 immobilised onto epoxy-agarose-UAB M2 derivative could be recycled up to 3 cycles, in the oxidation of chlorolactol, improving the biocatalyst yield and the product yield 1.5-fold. However, the use of soluble ADH99 and NOX resulted in the best option to perform the chlorolactol oxidation.

- Different EUGO immobilised derivatives were recycled up to 5 reaction cycles in the synthesis of vanillin. EUGO-epoxy-agarose-UAB M2 was selected as the most stable operationally immobilised derivative where 3-fold improvement of the biocatalyst yield could be obtained.

- In the vanillin synthesis two reaction conditions were compared: Maximum stability (pH 7.5) (Cond A) and maximum activity conditions (pH 9.5) (Cond B). The method of H_2O_2 was improved and the need for solvent, eliminated, resulting in reduced reaction time (to 4h) and increased STY (6.5-fold), reaching 9.9 g P L^{-1} h^{-1}.

- The reusability of the EUGO-epoxy-agarose-UAB M2 immobilised derivatives allowed reactions to be performed up to 5 and 18 reaction cycles with the same biocatalyst charge, using conditions A and B respectively. Even though the operational stability using conditions B was reduced, the reduction of reaction time led to a 12.4-fold improvement in biocatalyst yield compared to the soluble form. Moreover, the conditions B are greener than conditions A. These promising results bring the implementation of this biocatalytic process to closer to industrial implementation.

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APPENDIX

8

Table 8.1: extinction coefficient (ε) of vanillin at 340 nm.

pH	ε
5.0	1.9
6.0	3.0
6.5	5.0
7.0	7.7
7.5	14.9
8.0	23.1
8.5	25.5
9.0	26.7
9.5	27.1
10.0	27.4

Calibration curves

GC – Chlorolactone

Concentration ratio = Concentration of analyte / Concentration of internal standard.

Area ratio = area of analyte / area of internal standard.

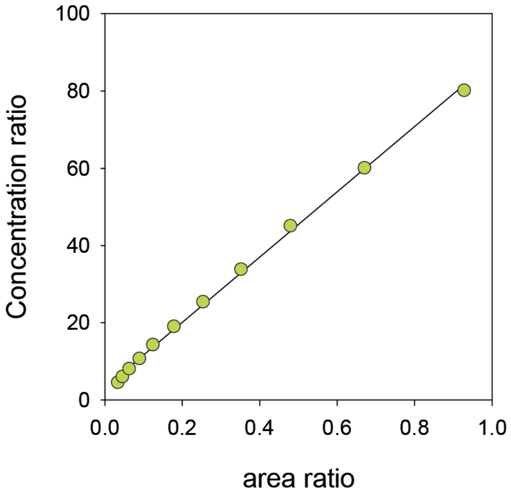

Figure 8.1: Calibration curve for Chlorolactone analysis by GC.

[Chlorolactone (mM) = [84.5352 · (area Chlorolactone/area IS) + 3.1808] / [IS] $R^2 = 0.9984$

Where IS is the internal standard, and R^2 is the coefficient of determination.

GC – Vanillyl alcohol

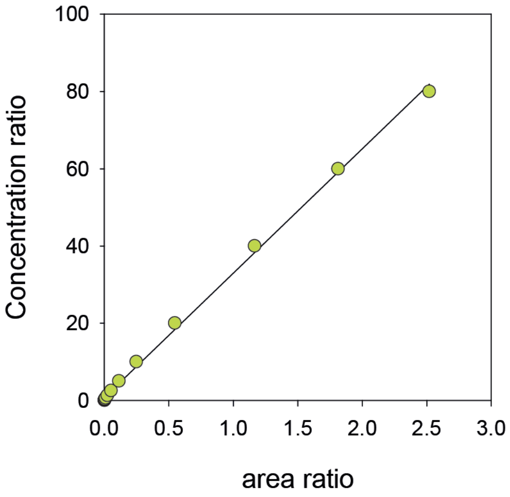

Figure 8.2: Calibration curve for vanillyl alcohol analysis by GC.

[Vanillyl alcohol] (mM) = [32.2165 · (area Vanillyl alcohol/area IS) + 0.7609] / [IS] $R^2 = 0.9983$

GC – Vanillin

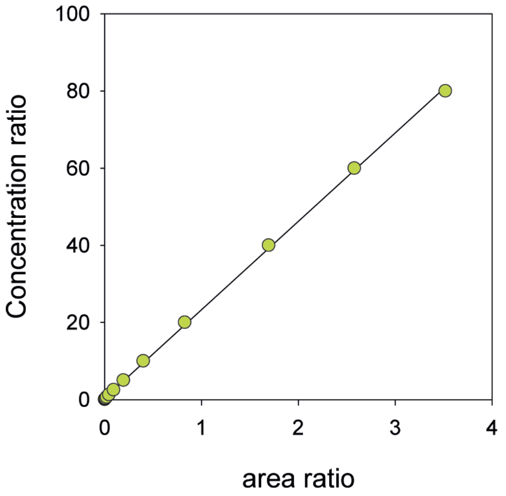

Figure 8.3: Calibration curve for vanillin analysis by GC.

[Vanillin] (mM) = [22.9279· (area Vanillin/area IS) + 0.3291] / [IS] $R^2 = 0.9996$

HPLC - Vanillyl alcohol

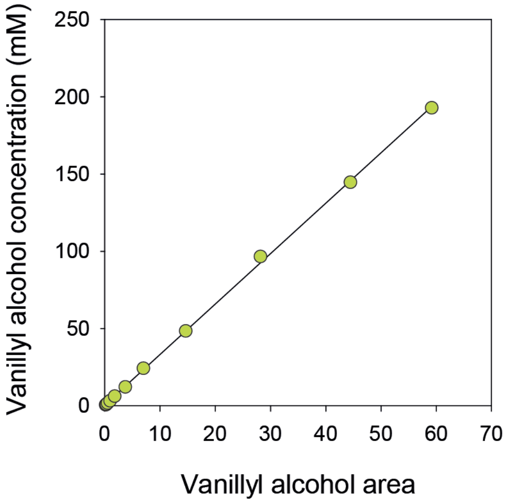

Figure 8.4: Calibration curve for vanillyl alcohol analysis by HPLC.

[Vanillyl alcohol] (mM) = 3.2660 · area Vanillyl alcohol + 0.5304 $R^2 = 0.9996$

HPLC – Vanillin

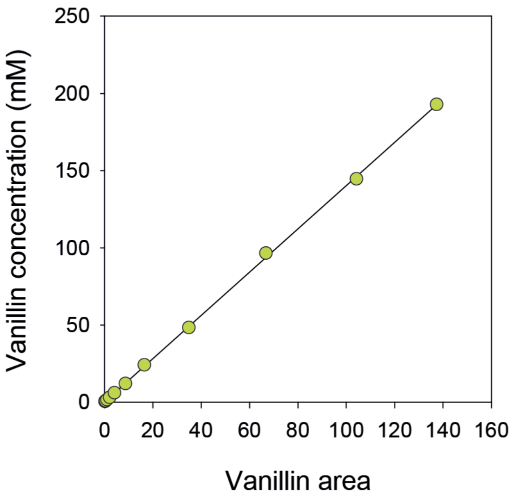

Figure 8.5: Calibration curve for vanillin analysis by HPLC.

[Vanillin] (mM) = 1.4009 · area Vanillin + 0.1597 $R^2 = 0.9997$

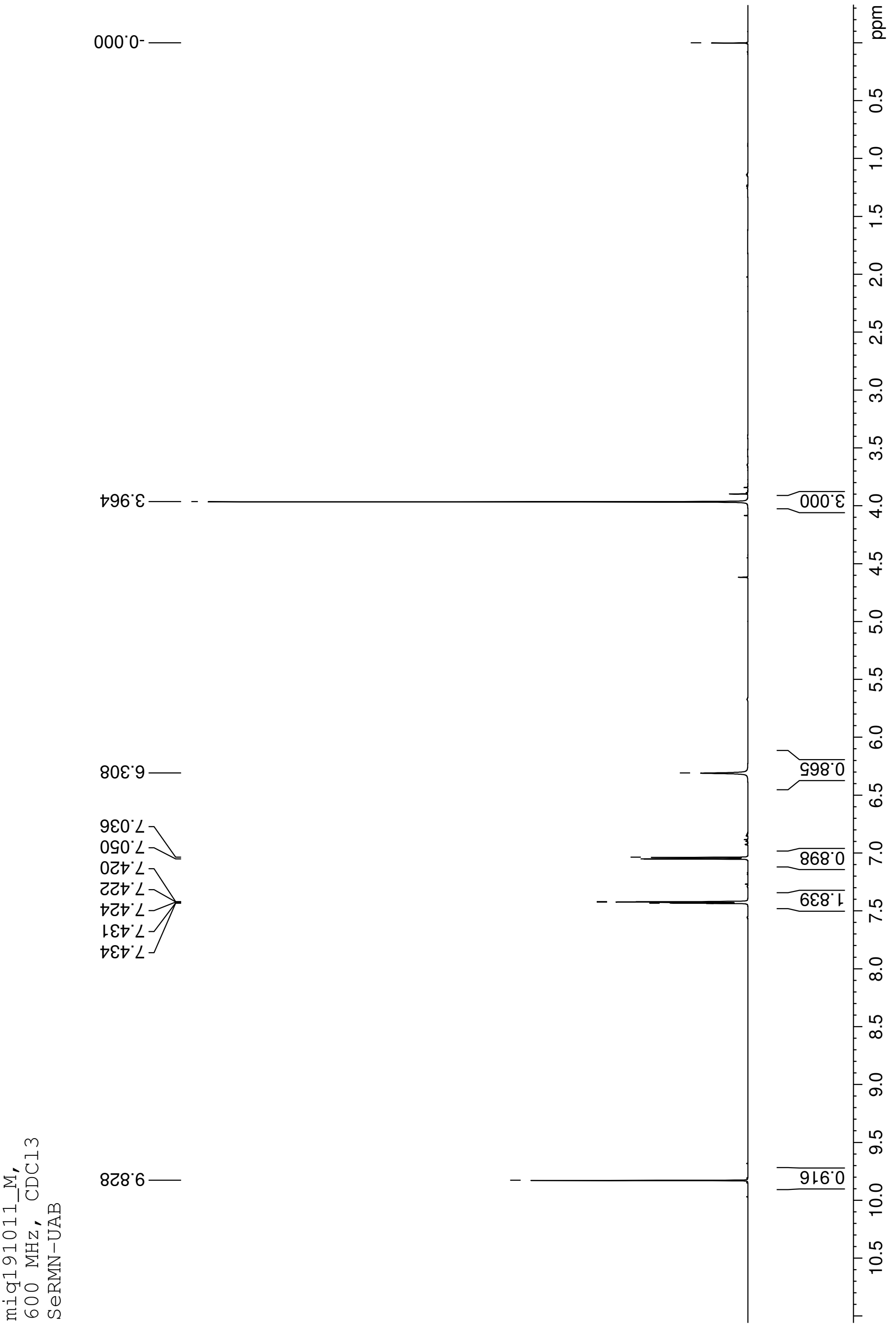

Figure 8.6: ^{1}H-Spectrum of the isolated sample obtained after purification of vanillin synthesis carried out at conditions B (Section6.2.2).

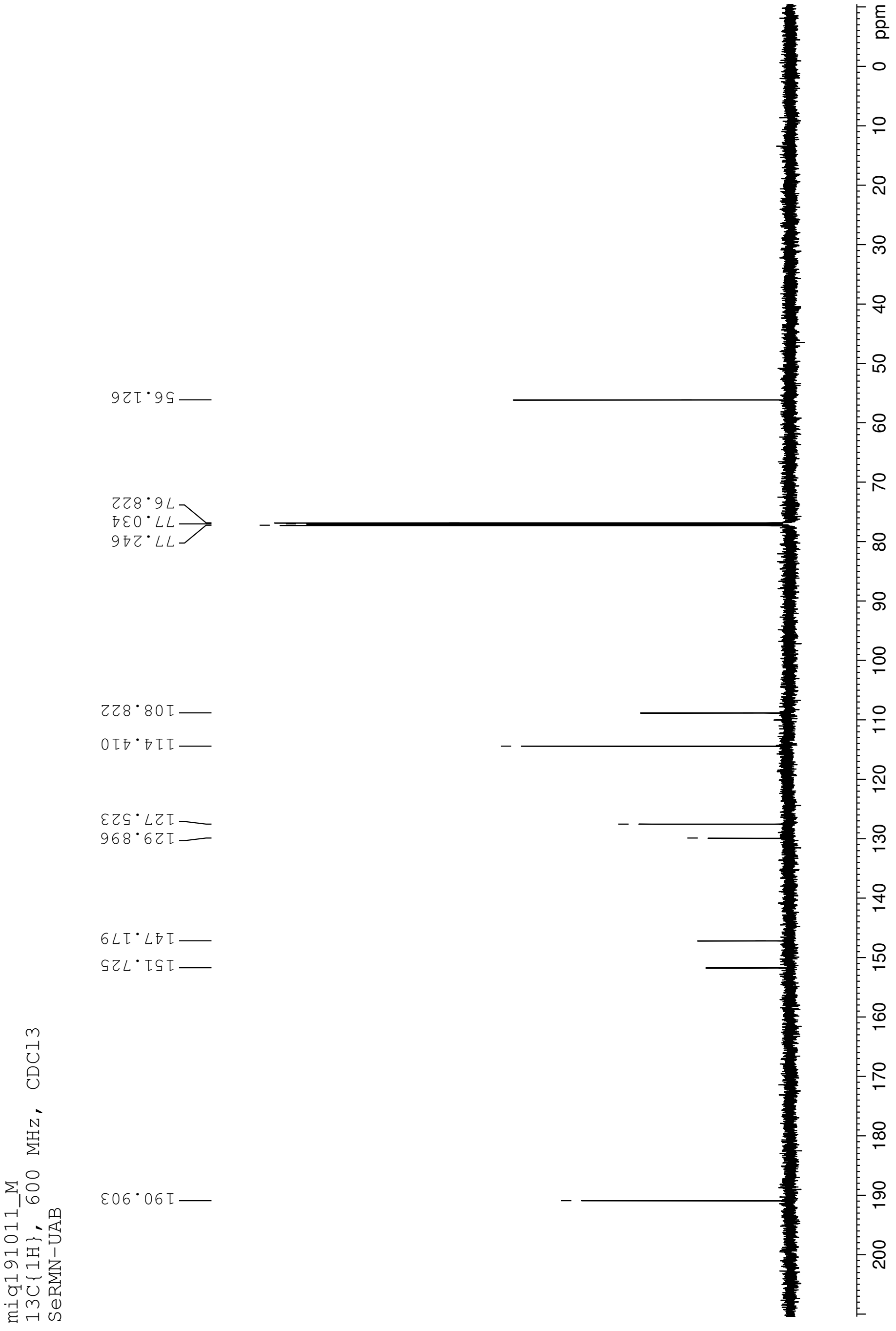

Figure 8.7: ^{13}C-Spectrum of the isolated sample obtained after purification of vanillin synthesis carried out at conditions B (Section6.2.2).

ACKNOWLEDGMENTS

9

9.1 Funding

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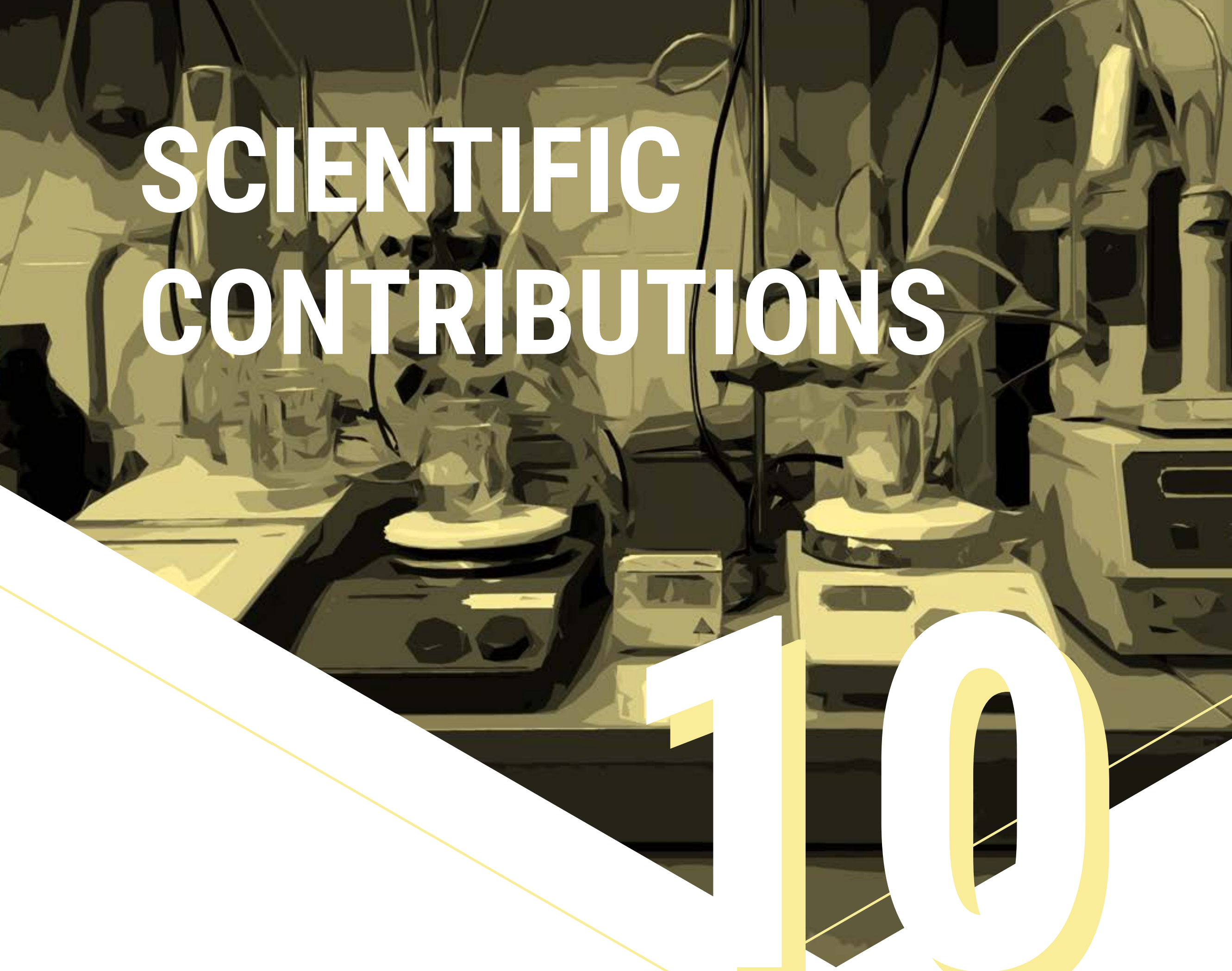

SCIENTIFIC CONTRIBUTIONS

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- Enzymatic synthesis of vanillin catalysed by an eugenol oxidase

M. García-bofill, P. W. Sutton, M. Guillén, and G. Álvaro
Appl. Catal. A, Gen., vol. 582, no. April, p. 117117, 2019.
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- Enzymatic synthesis of a statin precursor by immobilised alcohol dehydrogenase with NADPH oxidase as cofactor regeneration system

M. García-bofill, P. W. Sutton, M. Guillén, and G. Álvaro
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- Biocatalytic synthesis of vanillin by an immobilised eugenol oxidase: High biocatalyst yield by enzyme recycling

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- Biocatalytic Preparation of Vanillin Catalysed by Eugenol Oxidase

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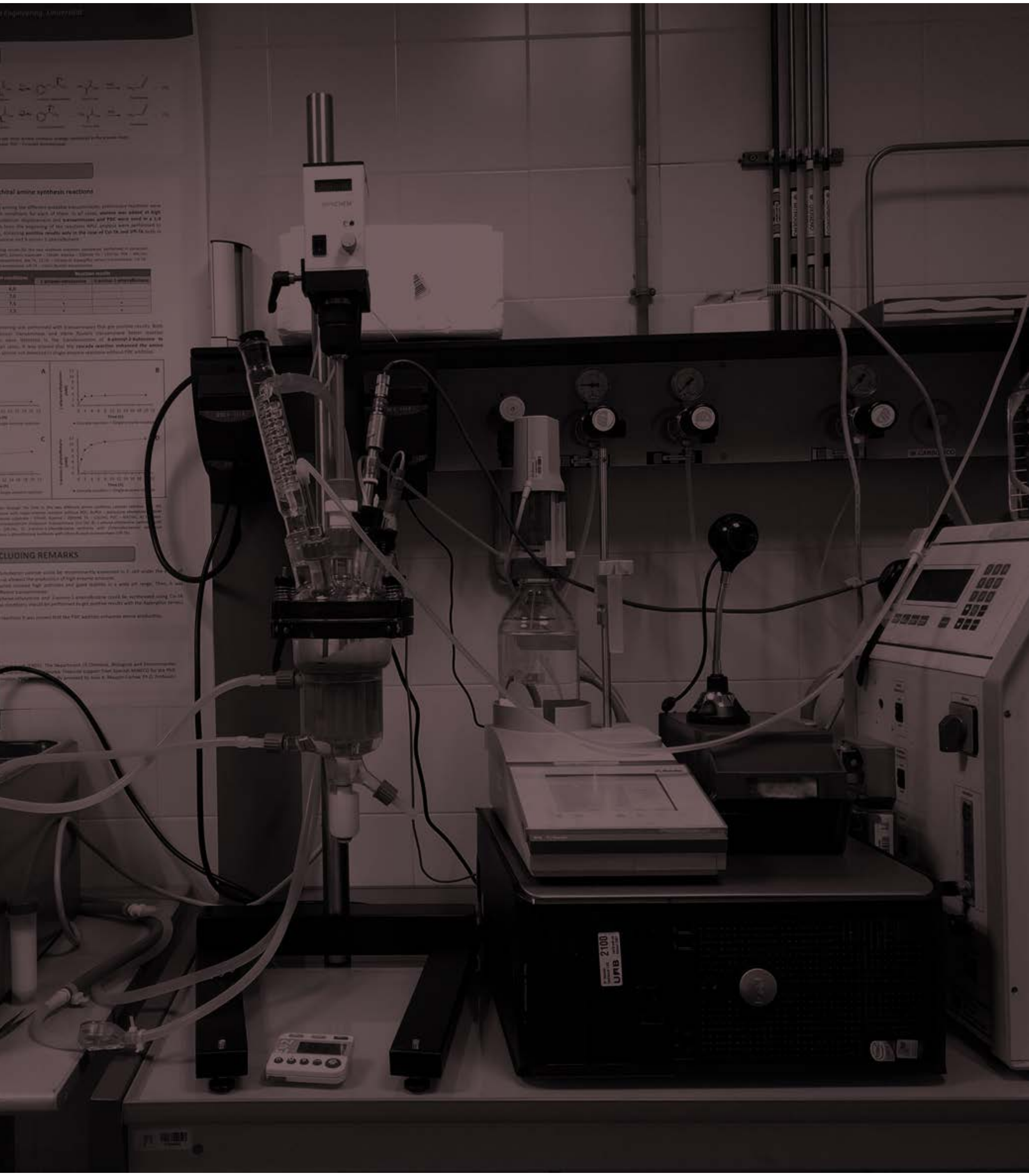
In: J. Whittall, P.W. Sutton, (Editors) Applied Biocatalysis: The Chemist’s Enzyme Toolbox, John Wiley & Sons, 2020.



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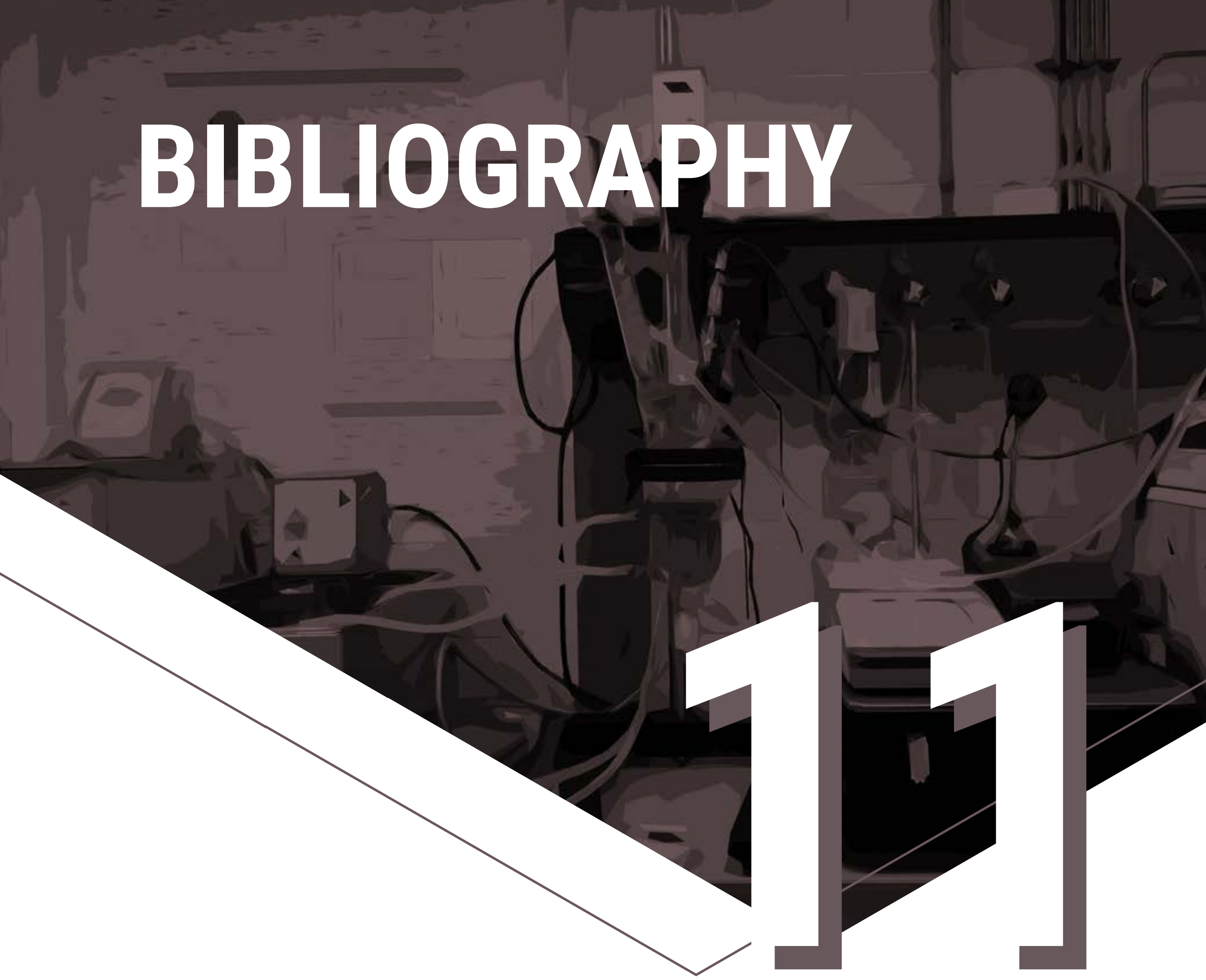

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