UNIVERSITÀ DEGLI STUDI DI MILANO

Ph.D. programme in CHEMISTRY

Department of Pharmaceutical Sciences (DISFARM)

XXXV Cycle

DEVELOPMENT OF SUSTAINABLE AND EFFICIENT TAILOR-MADE PROCESSES FOR PHARMA AND FOOD APPLICATIONS

CHIM 08

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A.A. 2021/2022

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Abbreviations

2-MeTHF: 2-methyltetrahydrofuran

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ACS: American Chemical Society

ADH: alcohol dehydrogenase

AE: atom economy

API: active pharmaceutical ingredient

AR: activity recovery

Asp: aspartic acid

Bet: betaine

BPR: back pressure regulator

CaLB: lipase from *Candida antarctica* B

CE: carbon efficiency

CED: cumulative energy demand

ChCl: choline chloride

CLEA: cross-linked enzyme aggregate

CPME: cyclopentyl methyl ether

CY: chemical yield

Cys: cysteine

DCE: 1,2-dichloroethane

DCM: dichloromethane

DES: deep eutectic solvent

DMA: *N,N*-dimethylacetamide

DMF: *N,N*-dimethylformamide

DMSO: dimethyl sulfoxide

DPPH: 1,1-diphenyl-2- picrylhydrazyl

e.e.: enantiomeric excess

Ec: *Escherichia coli*

EMY: effective mass yield

EPA: Environmental Protection Agency

EtOAc: ethyl acetate

FEP: fluorinated ethylene propylene

GC: gas chromatography

GCI: Green Chemical Institute

Glc: glucose

GVL: γ-valerolactone

HBA: hydrogen bond acceptor

HBD: hydrogen bond donor

His: histidine

homo-HTy: *homo*-hydroxytyrosol

HPLC: high-performance liquid chromatography

HTy: hydroxytyrosol

HTyAc: hydroxytyrosol acetate

i.d.: internal diameter

IC_{50}: half maximal inhibitory concentration

IL: ionic liquid

IME: immobilised enzymes

IR: infrared spectroscopy

IUBMB: International Union of Biochemistry and Molecular Biology

IWC: immobilised whole cells

LCA: life cycle assessment

LD_{50}: lethal dose 50%

MeCN: acetonitrile

Men: menthol

MeOH: methanol

MIC: minimum inhibitory concentration

MP: mass productivity

MsAcT: acyl transferase from *Mycobacterium smegmatis*

MW: molecular weight

NADES: natural deep eutectic solvent

NMP: *N*-methyl-2-pyrrolidone

NMR: nuclear magnetic resonance spectroscopy

Oct: octanoic acid

o.d.: outside diameter

OD_{530nm}: optical density 530 nm

Pa = Pseudomonas aeruginosa,

PBR: packed bed reactor

PDMS: polydimethylsiloxane

PEEK: polyether ether ketone

PEG: polyethylenglycol

PFA: perfluoroalkoxy alkanes

PG: propylene glycol

PME: process mass efficiency

PMI: process mass intensity

PS-BZA: polymer supported benzylamine

PTFE: polytetrafluoroethylene

PVA: polyvinyl alcohol

Rf: retention factor

RME: reaction mass efficiency

RMI: reaction mass intensity

Sa: *Staphylococcus aureus*

Se: S. enterica subsp. *enterica ser.* Enteritidis

Ser: serine

SI: solvent intensity

SP: specific productivity

STY: space time yield

TBME: *tert*-butyl methyl ether

TFA: trifluoracetic acid

THDES: therapeutic deep eutectic solvent

THF: tetrahydrofuran

TLC: thin layer chromatography

TTN: turnover number

Ty: tyrosol

TyAc: tyrosol acetate

VA: vanillic alcohol

VOC: volatile organic compounds

WI: wastewater intensity

YPD: Yeast Extract–Peptone–Dextrose

Introduction

"All sorts of things can happen when you're open to new ideas and playing around with things."

Stephanie Kwolek, Kevlar inventor

Humanity has always tried to understand the natural world. The need to know and explain intimately the essence of things seems to be inherent to mankind and chemistry, together with different discipline like philosophy, art and physics, is only one of the possible interpretations. The history of chemistry proceeds hand in hand with the development of society fulfilling both the desire of knowledge and the demand of improving life conditions. Chemistry is all around us and the capability to isolate or produce specific molecules has been not only a key factor for human development but also has also played a crucial role in the geopolitical chessboard. The fragile balance between the interest in the unveiling of internal mechanism of life and natural world and the craving of material possessions and power has been moved progressively towards the blind production of goods exceeding by far the real necessities of part of the world population and, at the same time, ignoring the needs of the poorest. The rushing industrial development which characterised the last centuries did not consider consequences for the environment and nature, bringing to the urgent needs today to find a different way to approach production. A global awareness towards the risk of a thoughtless exploitation of natural resources is becoming stronger every day while younger generations are becoming the ambassadors and the agents for a real and rapid change of direction.

Green chemistry

Scientists may depict the problems that will affect the environment based on available evidence, but their solution is not the responsibility of scientists but of society as a whole.

Mario Molina, 1995 Nobel Prize in Chemistry

The definition of green chemistry provided at the end of the last century from P. Anastas and T. Williamson reads as follows:

"Green Chemistry is an approach to the synthesis, processing and use of chemicals that reduces risks to humans and the environment."[1]

In the ground-breaking book co-authored by P. Anastas and J. Warner "Green Chemistry: Theory and Practice" (1998) the term gained formal recognition thanks to the declaration of the 12 principles of Green Chemistry which described a real path to follow for academia and industries in order to purse a more responsible scientific development (Figure 1).

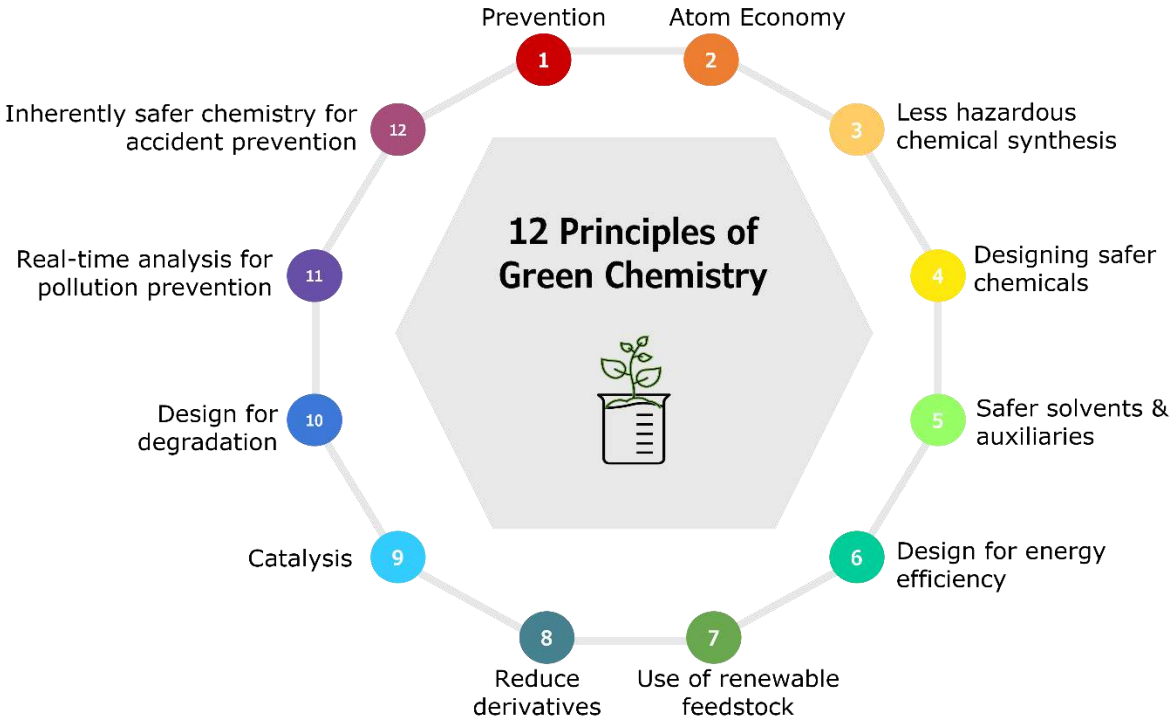


Figure 1. 12 Principles of Green Chemistry stated by P. Anastas and J. Warner [2].

Generally speaking, a green approach based on the twelve principles aims to:

- the reduction or, where possible, of waste and by-products together with the use of toxic reagents and solvents
- an efficient use of raw (preferably renewable) materials [3].

Green Chemistry Metrics

"When you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind"

Lord Kelvin

The scientific point of view could not ignore the quantification of the analysed phenomena; the measurability of an event is the foundation of the difference between the mere speculation and the understanding of a process and, consequently, the possibility to influence it. Green chemistry is not an exception and several metrics have been defined in the years to better comprehend the impact of synthetic practices. Experts discuss above which one is the most exhaustive and informative, but as L. Rogers and K. F. Jensen stated "which metric is the best is more a question of organization/personal preference"[4]. E Factor and Process Mass Intensity (PMI) are the two most common metrics employed, but most companies use more than one metric, in some cases as many as 10 metrics are compiled [5]. In the next paragraphs, some metrics will be presented in order to provide a general overview about the most used and discussed parameters adopted.

E Factor

According to R. Sheldon there is no doubt that the E Factor, or environmental factor, that he proposed back in the late 1980's, remains one of the most valuable parameters to consider in order to assess the sustainability of a process. The E Factor is defined as the ration between the amount of waste and the mass of product (usually expressed as kg/kg) [6].

$$E\ factor = \frac{mass\ of\ waste}{mass\ of\ product}$$

A low E Factor means a better ratio between the waste produced and the product: an E Factor of zero means that the best result has been obtained, no waste has been produced. Moreover, a lower E Factor is correlated with lower costs for APIs production, since is a direct image of lower process materials input, improved capacity utilisation, decreased costs of hazardous and toxic waste removal, and reduced energy demand [3].

Table 1 showing the E Factors for the different chemical industries published in 1992 is familiar to those who work in the field and had been the clear representation of how much could be done in the waste minimisation [7]. Pharmaceutical companies resulted to be the one which produce more waste compared to industries that have a notorious bad-reputation like oil-producing companies, highlighting once again how much important is a correct evaluation of the impact of synthetic procedures.

Industry Sector	Tonnage	E Factor (kg waste/kg product)
Oil refining	10^5-10^8	<0.1
Bulk chemicals	10^4-10^6	<1-5
Fine chemicals	10^2-10^4	5 to <50
Pharmaceuticals	10-10^3	25 to >100

Table 1. E Factors in chemical industries as reported by R. Sheldon in 1992.

The E Factor takes into account the product yield as well as waste from all of the auxiliary components and could be applied to the whole process [6].

Process Mass Intensity

As stated at the beginning of the chapter, it must be clear that there is not a definitive metric which define the greenness of a process, like a universal recognised truth. Despite the fierce advocacy of E Factor made by R. Sheldon, Process Mass Intensity (PMI) appears to be used by 67 % of chemical companies while the E Factor is used only by 48 % [5]. This parameter was introduced in 2006 by Environmental Protection Agency (EPA) and ACS Green Chemical Institute but it could be considered an evolution of the Mass Intensity defined by E. Heinzle, which was defined as the ratio between total input material and final product. PMI considers all materials involved in a single step or in a process, including water or workup chemicals. Energy consumption, safety and environmental impact are not considered [8–10].

$$Process\ Mass\ Intensity = \frac{total\ mass\ input\ materials}{total\ mass\ product}$$

A relevant information emerged by an analysis of PMIs from the ACS Green Chemical Institute is that solvents and water make up 56% and 32% by mass of the process waste, while raw materials account for 8% of overall process waste [11]. The crucial role played by the solvents in the ecological footprint of chemical process will be further analysed in the following paragraphs.

Atom Economy

The beginning of the 90's brought another yardstick which has to be considered when we speak about Green Chemistry. The Atom Economy (AE) was defined by B.M. Trost in 1991 as "*how much of the reactants end up in the product*" and is calculated dividing the molecular weight of the product by the total sum of the molecular weights of the raw materials and reagents [10], [12].

$$Atom\ Economy = \frac{MW\ of\ product\ x\ 100}{MW\ raw\ materials + MW\ reagents}$$

Atom Economy could be described as an attempt to define the reaction efficiency and could be a useful tool to quickly evaluate the resource efficiency together with the amounts of waste that will be generated in alternative processes, earlier that the actual running of any experiments. On the other hand, AE could be applied only to individual steps and implies that the exact stoichiometric quantities of starting materials will be used and a chemical yield of 100% will be obtained, while substances that are not present in the stoichiometric equation are ignored [6].

Reaction Mass Efficiency and Carbon Efficiency

Reaction Mass Efficiency (RME) could be considered an elaboration of AE since it takes into account the real quantities of reactants used and the chemical yield of the product. In contrast with AE, RME could not be used for a preliminary comparison of different processes in an early stage of development until some experimental works have been performed [13].

$$Reaction\ Mass\ Efficiency = \frac{total\ mass\ of\ product\ X\ 100}{total\ mass\ of\ raw\ material}$$

The Carbon Efficiency (CE) is similar to RME but considers only carbon and is defined as the percentage of carbon in the reactants that remain in the final product [14].

$$Carbon\ Efficiency = \frac{total\ mass\ of\ carbon\ in\ product\ \times\ 100}{total\ mass\ of\ carbon\ in\ raw\ material}$$

Additional metrics employed in the evaluation of the efficiency of a process are summarised in the following table (Table 2) adapted by a work of F. Roschangar and colleagues.

Metric	Formula	
Chemical Yield (CY)	$\frac{m\,(product) \times MW\,(raw\ material) \times 100}{MW\,(product) \times m(raw\ material)}$	%
Process Mass Efficiency (PME)	$\frac{m\ product \times 100}{total\ m\ of\ input\ materials\ included\ water}$	%
Mass Productivity (MP)	$\frac{m\ product \times 100}{total\ m\ of\ input\ materials}$	%
Effective Mass Yield (EMY)	$\frac{m\ product \times 100}{total\ m\ of\ raw\ materials + total\ m\ of\ reagents}$	%
Reaction Mass Intensity (RMY)	$\frac{total\ m\ of\ raw\ materials + total\ m\ of\ reagents}{m\ product}$	kg/ kg
Solvent Intensity (SI)	$\frac{total\ m\ of\ solvents}{m\ of\ products}$	kg/ kg
Wastewater Intensity (WI)	$\frac{total\ m\ of\ process\ water}{m\ of\ products}$	kg/ kg

Table 2. Common green metrics for the determination of process efficiency [10].

FOCUS

Life Cycle Assessment

In the view of a more complete analysis of the ecological impact of a chemical process, Life Cycle Assessment (LCA) resulted to be appealing for pharmaceutical companies. LCA is not a simple parameter but a methodology which allows to assess the potential environmental impacts and resources used throughout a product's life cycle [15].

Defined originally in 1969 at the Coca Cola company by Teasley and elaborated with the MRI, the Midwest research Institute, it was applied to understand which was the best option between the use of plastic bottles and cans through the quantification of “the energy, material and environmental consequences of the entire life cycle of beverage cans from raw materials extraction to disposal”[6], [16]. The basic idea of LCA is that the environmental impact related to a product must be assess, starting from the providing of the raw material to the end of the life of the product. LCA could be performed with two different approaches:

- Cradle-to-gate, which considers the journey from the raw material extraction and the manufacturing processes needed for the final product until it leaves the factory. This approach could be interesting, for example, to compare two different synthetic routes for the production of the same API.
- Cradle-to-grave, which also considers the use of the product and the management of its end of the lifecycle [16-17].

Solvents

"*The best solvent from an ecological point of view is without a doubt no solvent"*

Jürgen O. Metzger, "Solvent free organic synthesis"[18]

The description of the most common yardsticks used in the determination of the efficiency and ecological impact of a chemical process has highlighted the significant role played by solvents, which could substantially increase the environmental footprint. Organic chemists, and in particular synthetic chemists in the pharmaceutical fields, are devoted to the development of the best synthetic procedure focusing their attention on the final product, its purity, consistent quality, and high yields. However, solvents are not just the media where the reaction took place, allowing reactants and catalyst to get in contact, but are also employed in the purification's steps (*i.e.*, extractions, crystallisation, chromatographic separations), in the analytical phases and in the cleaning procedures. All these needs lead to the fact that in a classical batch (non-polymeric) chemical approach, like the one commonly applied by pharmaceutical companies, the 80-90% of the total mass in the process is represented by solvents (30% water and 60% organic solvents) [19].

Since solvents are the larger part of the mass utilisation, they could be considered the main characters when we speak about the toxicity of a chemical process: they are often volatile, flammable, toxic or and/or corrosive. Moreover, their characteristics did not comprise only the environmental wellness (air and water pollution above all), but they also seriously affect the health of the operators [20].

The statistics report that are commonly produced between 25 kg and 100 kg of waste generated by solvents to produce 1 kg of API [21]. The estimation is that solvents represent around the 60% of all industrial emissions and 30% of all volatile organic compound emissions worldwide [22]. Furthermore, not all solvents are the same: some are worse than others. A study of GSK pointed out the ten most used solvents (>80% of the time) by the company in the years between 1990-2000 and the change in their application in pilot plan in 2005 (Table 3). Even if the trend showed a decreasing in the use of toluene, dichloromethane, and tetrahydrofuran, their employment was still considerable and not sufficient for a significant change of direction [19].

	2005 Rank	1990- 2000 Rank
2- Propanol	1	5
Ethyl acetate	2	4
Methanol	3	6
Denatured ethanol	4	8
n-Heptane	5	12
Tetrahydrofuran	**6**	**2**
Toluene	**7**	**1**
Dichloromethane	**8**	**3**
Acetic acid	9	11
Acetonitrile	10	14

Table 3. GSK ranking of solvent used in pilot process prior 2000 and in 2005 [19].

In fact, dichloromethane, together with chloroform, are solvents whose use has been restricted by the European regulation concerning the 'Registration, Evaluation, Authorisation and Restriction of Chemicals" (REACH), due to major safety hazards, making a change of direction unavoidable (Table 4)[23].

Solvent	Safety hazard	Pictogram
Benzene	• Affects the bone marrow that cause anemia, leukopenia, and thrombocytopenia • Long exposure leads to leads to aplasia and pancytopenia	
Chloroform	• Hepatotoxic and nephrotoxic • Carcinogenic	
Dichloromethane	• Skin irritation • Potential human carcinogen	

Table 4. Safety risks associated with toluene, chloroform, and toluene.[24].

Notwithstanding that the best solvent appears to be no solvent at all, neat chemistry is not always an accessible path. For this reason, more practical ways to approach the problem are pursued at the moment by following four main directions:

1. SUBSTITUTION

   the easiest approach is to replace hazardous solvents with ones that show better EHS (environmental, health and safety) properties.

2. BIOBASED SOLVENTS

   use of ‘‘bio-solvents’’, *i.e.,* solvents produced with renewable resources, to avoid the use of fossil resource and fossil fuel CO_2 emissions to the environment.

3. SUPERCRITCIAL FLUIDS

   supercritical fluids are environmentally harmless (*e.g.,* the use of supercritical CO_2).

4. NOVEL SOLVENTS

   ionic liquids, deep eutectic solvent and natural deep eutectic solvents, which show low vapour pressure, and thus less emission into the air [25].

In the following paragraphs the replacement of solvents, solvent from renewable sources and novel solvents will be discussed in detail insofar as a key focus of this doctoral project.

FOCUS

What is a Green Solvent?

Once established that solvents are linked to a series of health, safety and environmental hazards, remaining at the same time crucial for the production of chemicals of different type, the issue is clear: how can we determine the solvent that is less harmful? In few words, how can we say that a solvent is "green"? First, in order to compare different solvents, it is necessary to determine a score, a value (once again a mathematical measurement) which allows to establish a ranking and an objective comparison between different substances. Several methods are reported in literature, but a significant answer to this question has been proposed by the ETH of Zurich as a multi-stage assessment: the first one employs the environmental, health and safety (EHS) assessment method, whereas the second method is focused on the life-cycle assessment (LCA) method [25]. The EHS assessment method is a "screening method that identifies possible hazards of chemical substances in early phases of the chemical process design" in which the chemicals are assessed in nine effect categories which are summarized in Table 5. For every effect category, a score between zero and one is calculated, resulting an overall score between zero and nine for each substance [25–27].

EFFECT CATEGORIES	HAZRDS
Release potential	
Fire/explosion	Safety hazards
Reaction/decomposition	
Acute toxicity	
Irritation	Health hazards
Chronic toxicity	
Persistency	
Air hazard	Environmental hazards
Water hazard	

Table 5. Nine categories in EHS assessment [25].

In this framework, when the energy required to produce a solvent and the options available at end-of-life to recover some of that energy are considered, another sustainability indicator is designed and calculated: the net cumulative energy demand (CED) of solvent production (expressed in MJ kg^{-1}). The combination of low value of both these scores indicates a less harmful solvent for the environment and the health [27].

Solvent Selection Guides

The right choice of the solvent at the beginning of the development of a new synthetic route remains the best way to prevent the use of harmful substances because it is more complicated to apply any change when the process is robust, verified and already running. For this reason, several pharmaceutical companies like Sanofi, Astra Zeneca, Pfizer, and GSK itself have compiled solvent guide to better approach the solvent selection in each phase of the drug development [28–30].

Pfizer's Selection Guide

The guideline builds up by Pfizer was the first one to be published. It was a friendly user system where the solvents were classified in "preferred", "usable" or "undesirable", and for this last class a substitution guide was provided (Table 6) [23], [28].

Preferred	Usable	Undesirable		Alternatives
Water	**Cyclohexane**	**Pentane**	→	Heptane
Acetone	**Heptane**	**Hexane(s)**	→	Heptane
Ethanol	**Toluene**	***Di*-isopropyl ether**	→	2-MeTHF, TBME
2-Propanol	**Methylcyclohexane**	**Diethyl ether**	→	2-MeTHF, TBME
1-Propanol	**Methyl *t*-butyl ether**	**Dichloromethane**	→	Application dependant
Ethyl acetate	**Isooctane**	**Dichloroethane**	→	DCM
Isopropyl acetate	**Acetonitrile**	**Chloroform**	→	DCM
Methanol	**2-MethylTHF**	**Dimethyl formamide**	→	Acetonitrile
Methyl ethyl ketone	**Tetrahydrofuran**	***N*-Methyl pyrrolidone**	→	Acetonitrile
1-Butanol	**Xylenes**	**Pyridine**	→	Triethylamine
***t*-Butanol**	**Dimethyl sulfoxide**	**Dimethyl acetate**	→	Acetonitrile
	Acetic acid	**Dioxane**	→	2-MeTHF, TBME
	Ethylene glycol	**Dimethoxymethane**	→	2-MeTHF, TBME
		Benzene	→	Toluene
		Carbon tetrachloride	→	DCM

Table 6. Pfizer's guide for solvent selection and alternatives to the undesirable solvents [23], [28].

The interesting fact was that even if dichloromethane, an "undesirable" solvent, it was suggested as an alternative for other chlorinated solvents as chloroform, dichloroethane, and carbon tetrachloride, leading to a 50 % reduction in chlorinated solvent use over 2 years. Moreover, Pfizer observed a reduction in the use of *n*-hexane (neurotoxic) and *n*-pentane (volatile and flammable) in favour of the safer *n*-heptane, just thanks to the higher awareness spread among the researchers about the solvent issue [23].

GSK's Selection Guide

GSK employed its first selection guide back in 1999, implementing it in 2004 by adding life cycle assessment analysis. Then, in 2011, another revised version was published with, among other modifications, a significant increase in the number of solvents considered, from 47 to 110. In the last updated version published back in 2015, the number of solvents considered was further expanded with the addition of 44 new solvents, among them several novel solvents considered "green" by the literature [31]. The classical traffic light colour coding (already seen in the Pfizer list) was applied to the GSK solvent selection guide together with an overall summary score (Table 7) assigned to four main parameters: waste, environment, health, and safety. This score is defined as the geometric mean of relevant category scores calculated as the following equations show Figure 2.

Parameter	Equations for GSK solvent guide
Waste	$W = \sqrt[4]{I \times R \times BT \times VOC}$
Environment	$E = \sqrt{\text{Air} \times \text{Aqueous}}$
Health	$H = \sqrt{\text{Health hazard} \times \text{Exposure potential}}$
Safety	$S = \sqrt{F\&E \times R\&S}$

I incineration score, *R* recycle score, *BT* biotreatment score, *VOC* VOC emissions score

Figure 2. Equations for GSK solvent guide

GSK Colour assignments for summary & category scores	
Score < 3.5	**RED**
3.5 ≤ score ≤7.5	**AMBER**
7.5 ≤ score	**GREEN**

Table 7. GSK solvent classification [31].

Moreover, a composite score was calculated as the geometric mean of the value associated to the four previous general areas (waste, environment, health, and safety) to establish a general ranking of the solvents [31]. In the next page, the last selection guide of GSK (Table 8) is shown in order to have a visual comparison between the one proposed by Pfizer, an American industry, and the one elaborated by the British company.

Table 8. GSK Solvent Sustainability Guide [31].

Sanofi Guide

The French pharmaceutical giant has also developed its own solvent selection guide, based on the project Sanofi Aventis HSE (Health, Safety & Environment) dividing the solvents into two main categories: recommended (A list) and to be replaced (B list). Even if the company was part of the Green Chemical Institute Pharmaceutical roundtable, it did not participate in the elaboration of the ACS-GCIPR solvent guide, opting for an internal guide. However, this guide was defined as not "user friendly" from the same company (it was in alphabetical order and B list was too long and difficult to use), reason why a working group was created in 2009 in order to redesign and update this guide [30]. In this guide, colour codes are associated with some data, resulting in the following prescription:

Colour	Recommendation
GREEN	Recommended Solvent (most of them are in the A list of HSE guide)
YELLOW	Substitution advisable. They could be use on industrial scale with some constrains
RED	Substitution requested. These can still be used in the pilot plant, but their use on industrial level for new processes has to be justified on the basis of unsuccessful substitution experiments.
BROWN	Banned, even at laboratory scale for safety, health, and environmental reasons.

Table 9. Sanofi Aventis colour code for solvents.

However, if we also consider the evaluation made by other pharmaceutical companies like AstraZeneca or the one elaborated by the same American Chemical Society (ACS) and Green Chemical Institute Pharmaceutical Roundtable (GCI-PR) in collaboration with different companies, it is possible to observe two things: the first one is that there will not be two identical selection guides as minor and not so minor differences in the cataloguing of solvents will be present based on the different definitions of greenness and methods to asses this value, that is not an intrinsic property of a substance but could be susceptible of different considerations (*e.g.*, methyl ethyl ketone ranked by Pfizer as preferred and by GSK as a solvent with some known issues). Secondly, water is always the less harmful choice compared to classical organic solvents, but also to novel eco-friendly solvents, bringing an important issue on the table: performing chemical reactions with organic molecules in aqueous environment, a matter that has already a perfect solution in the use of biocatalysis.

Green and Sustainable Solvents

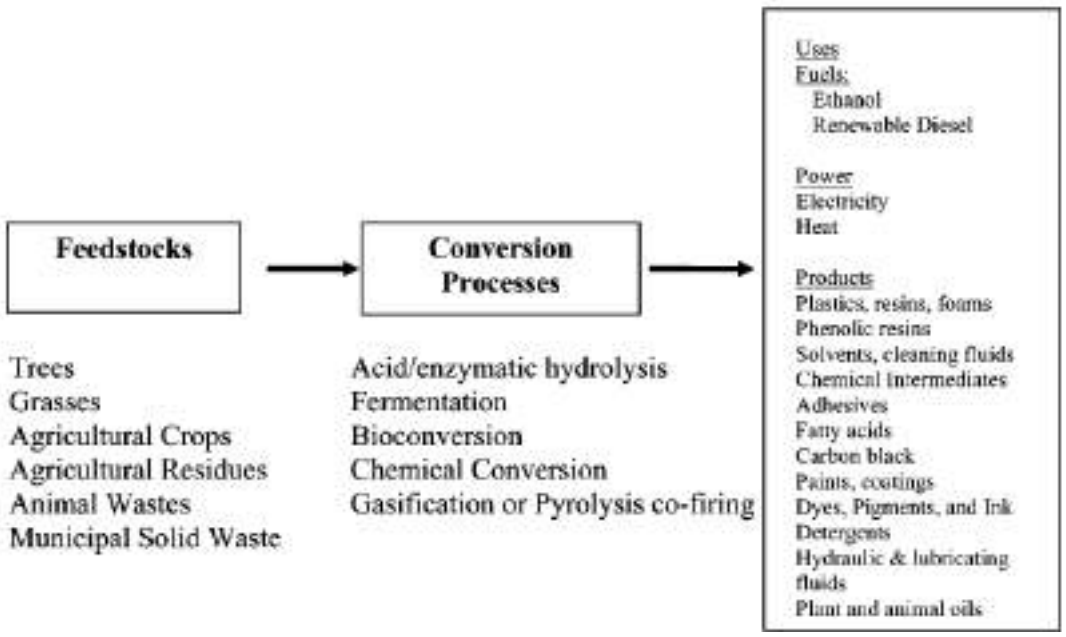
Before moving to a better explanation on how and when is possible to employ water as reaction environment, a devoted paragraph is absolutely necessary to describe which are the less impactful and more sustainable options in the solvent field, with a particular focus on the novelty advised by the recent scientific literature. How to define a solvent green or not has been already discussed in this chapter, together with the common approach to replace a harmful organic solvent with a less detrimental one. Anyway, this is not the only way to approach the urgent issue represented by the use of solvents. The design, production, and use of neoteric solvents, *i.e.*, biobased solvents, deep eutectic solvents, and ionic liquids, are also valid answers to the problem constituted by the ecological impact of common organic solvents.

Biobased solvents

Fossil based economy has already showed its limits and side effects that are currently affecting our everyday life. However, humanity depend on fossil fuels to produce the 80% of worldwide energy and the 90% of the chemicals [32-33]. The employment of renewable feedstock is one of the principles of Green Chemistry and it has been already applied to the production of fuels, high value chemicals and other materials. In order to obtain these valuable products from biomass, a system similar to a petroleum refinery is necessary, commonly called a biorefinery. The concept is that inside a biorefinery biological masses derived from agricultural commodities (*i.e.*, biomass) are the input materials that will be transformed, thanks to complex and different technologies, into multiple products. The biorefinery feedstocks include grasses, starch crops (wheat and maize), sugar crops (beet and cane), lignocellulosic crops, lignocellulosic residues (stover and straw), oil crops, aquatic biomass (algae and seaweeds) and organic residues (industrial, commercial, and post-consumer waste) [32].

Scheme 1 reports the general flow stream of a biorefinery, from the input material, through the different conversion processes and to the final valuable products. The products coming from biorefinery could be divided in two main area:

- High-value low-volume products (HVLV): products of high added value compared to the starting material which increase the profitability of the process.
- Low-value high-volume products (LVHV): this kind of intermediates are converted into energy to meet the global energy demand [33].



Scheme 1. Biorefinery process [33].

The production of solvent has not been excluded by this virtuous trend [27]. Biobased solvents are produced from biomasses derived from vegetable, animal, or raw materials. Since one of their major goals is to be sustainable, they need to show greener features compared to classical organic solvents, like a VOC reduction, biodegradability and no-ecotoxicity, together with a better safety profile.

The major classes of biobased solvents are:

- esters of natural organic acids (produced by fermentation of renewable materials)
- fatty acid esters
- bioethanol
- terpenic compounds
- isosorbide
- glycerol derivatives [34].

There are three main methods to produce bio-based solvents: through fermentation, chemical transformation of the biomasses, or using waste material from other processes [27]. In the following paragraphs, some relevant and highly employed renewable solvents will be discussed with further details.

Glycerol

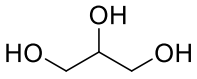
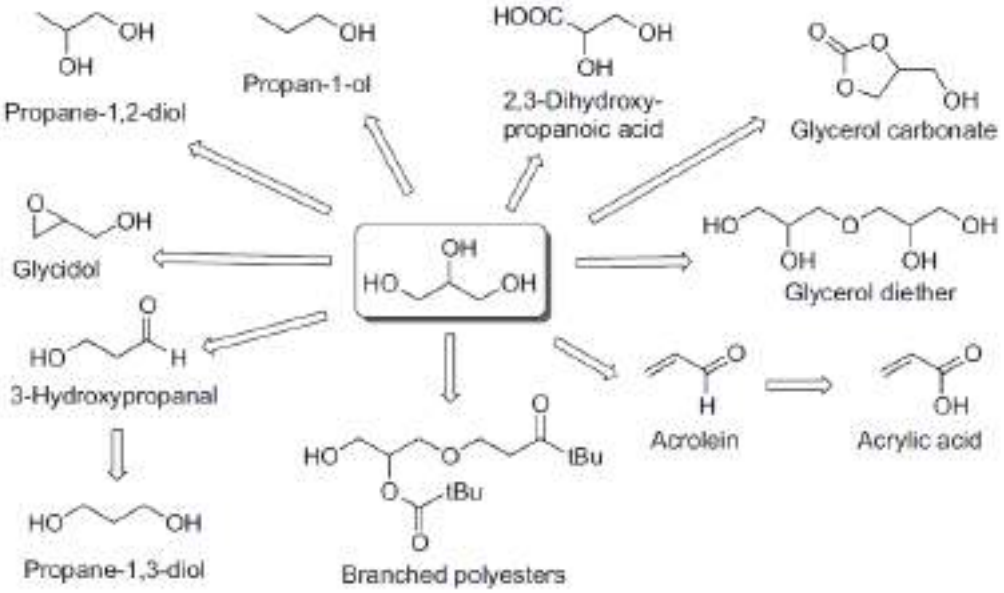


Figure 3. Glycerol chemical structure.

Glycerol is a biobased alcohol, available in bulk quantities from the waste generated by the biodiesel industry (around 10% of the total output). More than 1500 uses of glycerol are known including applications as an ingredient or processing aid in cosmetics, toiletries, personal care products, pharmaceutical formulations, and food industry. In addition to its property as a green solvent, glycerol is well known as a renewable starting point to produce several relevant fine chemicals (Scheme 2) [35-36].



Scheme 2. Fine chemicals derived from glycerol [27].

Glycerol possesses several favourable characteristics which made it a green solvent: it is cheap (0.50 €/Kg for pharmaceutical grade 99.9% in 2010), safe (LD_{50} oral rat = 12600 mg Kg^{-1}), non-flammable and biodegradable; moreover, it has a low vapour pressure and a high boiling point (290 °C), it is stable under usual storage conditions, and is highly polar. Glycerol has similar behaviour to water, DMSO and DMF, but it allows to solubilise substances that are poorly soluble in water, and it facilitates the solubilisation of inorganic salts, acids, bases, enzymes and many transition metal complexes. Due to its immiscibility with several classical organic hydrophobic solvents, it is possible to collect the reaction products by simple liquid–liquid phase extraction. Moreover, the

high boiling point allows to consider distillation as a feasible technique to collect final product and enable to perform processes at high temperatures. There are also some disadvantages that has to be taken into account: the high viscosity that could hinder a correct mass transfer (but it must be considered that at temperature above 60 °C the viscosity of glycerol decrease), the chemical reactivity of the hydroxyl groups that could lead to the formation of side products, and its coordinating properties which may cause some problems when transition metal complex catalysts are used [37]. Nevertheless, its use in organic synthesis is considerable, since it improves reaction performances and selectivity, product separation and effective catalyst recycling [38]. Glycerol could also be an alternative to conventional solvents for many organic catalysed and non-catalysed reactions such as hydrogenations, Heck, Suzuki, or aza-Michael reactions [39-40].

γ-Valerolactone

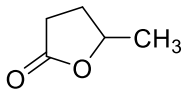


Figure 4. γ-Valerolactone chemical structure.

γ-Valerolactone (GVL) is considered a platform chemical that is a substance which could act as the substrate to produce several high value chemicals [32]. It could be used as a solvent, as a fuel itself or as an additive to current petroleum fuels, as a building block in the production of polymers, as an intermediate in the fine chemical synthesis and as a flavouring agent in perfumes and foods thanks to its sweet, herbaceous odour [41]. GVL is usually derived from levulinic acid or alkyl levulinates, which are obtained from lignocelluloses or carbohydrates through hydrolysis or alcoholysis in water or alcohol in the presence of acid catalysts. Scientific literature reported several synthetic procedures to obtain GVL [42-43]. This five-carbon cyclic ester possesses a low toxicity (LD_{50} oral-rat = 8800 mg kg^{-1}), is miscible with water, stable under normal storage conditions, does not form peroxides in air, and has a high boiling point (207-208 °C). However, GVL has a vapor pressure greater than 10 Pa, which can make it dangerous due to VOC emissions [41]. GVL could be employed as a greener option to replace dipolar aprotic solvents, especially in cross-coupling reactions (*e.g*, Hiyama, Sonogashira, Mizoroki–Heck reactions) [44–46].

2-Methyl tetrahydrofuran

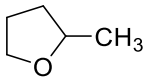
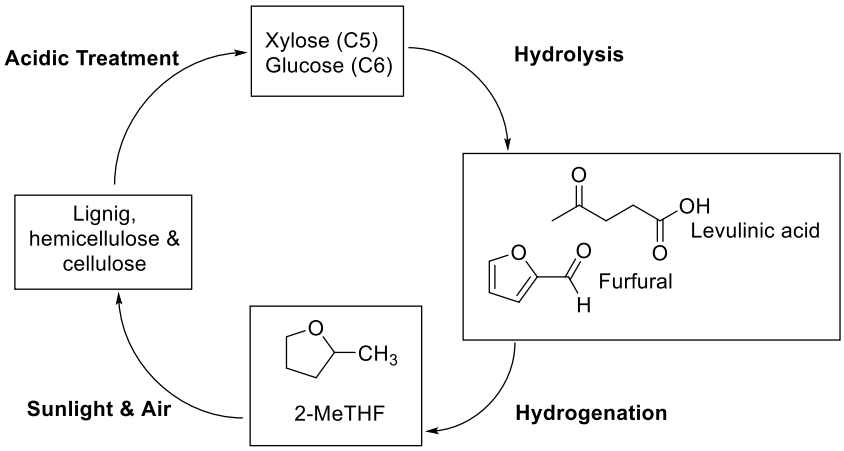


Figure 5. 2-Methyl tetrahydrofuran chemical structure.

Cellulose, hemicelluloses, and lignin contain sugars as glucose and xylose that could be converted in levulinic acid and furfural with acidic treatment, obtaining the substrates for the production of 2-methyl tetrahydrofuran (2-MeTHF). An interesting point is that 2-MeTHF could be abiotically degraded by sunlight and air (Scheme 3), even if the mechanism is not already clear (probably via oxidation and ring-opening) [47-48].



Scheme 3. Conceptual scheme of 2-MeTHF life cycle.

Originally design as a biofuel, 2-MeTHF is now employed as a bio-based alternative to tetrahydrofuran (THF). Compared to THF, 2-MeTHF has a lower miscibility with water that allows a full recovery of the solvent and a higher boiling point (80 °C, compared to 60 °C) that is favourable to perform reaction at higher temperature, all combined with a higher stability. However, it is a strong Lewis base compared to THF. 2-MeTHF is an aprotic solvent that resembles toluene in terms of physical properties and could be used to replace not only THF and toluene, but also highly chlorinated solvents including DCM and DCE [49]. These features make possible to use this solvent in synthetic procedure involving organometallics, organocatalysis, and biotransformations, both in industry and academia [47], [50]. Furthermore, 2-MeTHF has an increased tolerance respecting THF both in acids (*e.g.,* HCl) and in a base, making it easy to recycle the solvent [47]. The low volatility and high flash point make it a safer solvent for industrial processes. Moreover, the toxicological study claims that exposure of the solvent is not associated with any genotoxicity and mutagenicity [48].

Cyrene

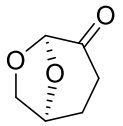
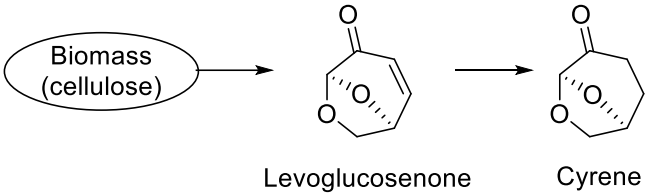


Figure 6. Cyrene chemical structure.

Dipolar aprotic solvents are a class of solvent largely employed in synthetic chemistry (*e.g.*, *N*,*N*-dimethylformamide, DMF, and *N*-methyl-2-pyrrolidone, NMP), but usually they are produced from not renewable sources and are classified by almost all solvent selection guides as "undesirable" solvents. For this reason, a huge effort has been made to find ecological alternatives, like the already described GVL, in order to replace this type of solvents. Dihydrolevoglucosenone (*i.e.*, Cyrene ™) is a bio-based solvent obtained from cellulose through a two steps process (Scheme 4) [51].



Scheme 4. Two-step process to produce Cyrene ™ [51].

Cyrene ™ is relatively stable in the presence of weak acids and unstable with oxidants [27]. A preliminary Ames mutagenicity screening has been completed on Cyrene ™ with no mutagenicity observed, however it has been given a hazard warning for being an eye irritant (E319) [51-52]. It has a high boiling point (227 °C), and from the extensive work about Cyrene ™ written by Dr Jason Camp in was found that "*comparing the Kamlet–Abboud–Taft and Hansen solubility parameters, Clark and colleagues found that it had dispersion parameters closest to DMSO (18.8 vs. 18.4 MPa), polarity closest to DMA (10.6 vs. 11.5 MPa), and hydrogen bonding-like interactions that were most similar to NMP (6.9 vs. 7.2 MPa)*" [51], [53]. For these reasons, Cyrene ™ could substitute not only DMF and NMP, but also DMSO and DMA. The solvent properties of Cyrene ™ have been already exploited to perform different chemical procedures, *e.g.*, urea synthesis, amide synthesis from acid chlorides and amines, HATU-mediated amide coupling, and cross coupling reactions [54–58]. However, it must be considered that Cyrene ™ is also an interesting scaffold for the synthesis of different chemicals, reason why its reactivity (it reacts in the presence of hard nucleophiles, acids, peroxides, nucleophilic primary amines and strong bases) must be considered when used as a solvent [51].

Neoteric solvents: Deep Eutectic Solvents

Deep eutectic solvents are mixture of two or more compounds, a hydrogen bond donor species (HBD) and a hydrogen bond acceptor species (HBA), that when mixed together in fixed molar ratio show a significant depression of their melting point, caused by the formation of an eutectic mixture characterised by a melting point below 100 °C. Some clarification before starting. After their appearance on the scientific scene, some generalisations about DESs have brought life to some "urban legends" that created wide misconceptions. DESs are not pure novel compounds, but always mixtures. They are related to ILs since they have common properties, but they are not the same thing: ILs are fluid constituted solely by ionic species, while DESs can be at the best solutions of ions. Moreover, the general statement is that DESs are cheap, green, and eco-friendly solvents but also this part needs an explanation note since these characteristics largely depend on the substances of which a DES is made of. Lastly, to consider an eutectic mixture a "deep" eutectic mixture, Martins and co-workers defined that is necessary that "the eutectic point temperature is below that of an ideal liquid mixture, presenting significant negative deviations from ideality ($\Delta T_2 > 0$)", where ΔT_2 stands for the temperature depression which is the difference between the ideal and the real eutectic point, reason why to identify correctly a DES its phase diagrams should be known [59].

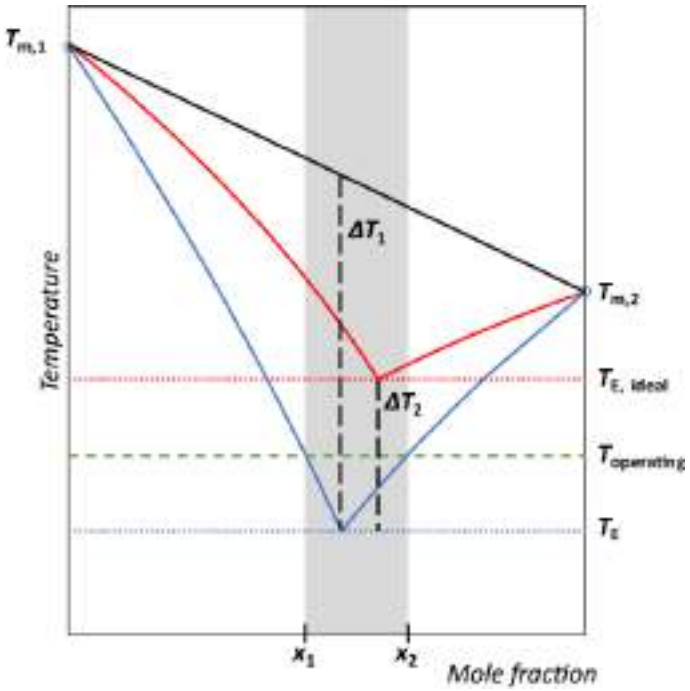


Figure 7. Schematic representation of the comparison of the solid-liquid equilibrium phase diagram of a simple ideal eutectic mixture (red line) and a deep eutectic mixture (blue line) [59].

The first description of DESs was given by Abbott and colleagues at the beginning of the XXI century, which described a novel solvent made by the eutectic mixture of urea and choline chloride, liquid at room temperature [60]. Since then, the number of scientific publications related to DESs has increase, with 514 papers published in 2021 based on the statistics reported by PubMed (**Figure *8***) [61].

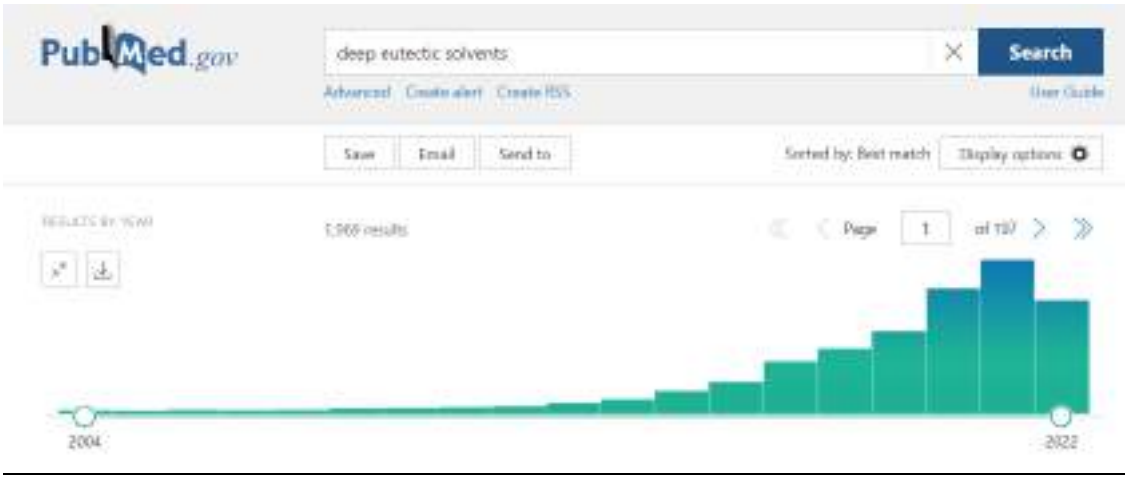


Figure 8. DESs timeline based on PubMed.

The increasing interest on the topic found several reasons, starting from their properties. The preparation of a DES is easier compared to the preparation of an IL. To prepare an IL, it is necessary to dilute, mix and stir the components, afterwards the reaction mixture is purified through the separation of the solid residue and washed with a solvent (that is a relevant issue when the ecological impact of a procedure is considered). For different components the time changes, but also the solvent used, and the temperature employed: generally, the process could reach up to 48 hours and the temperature range varies from 25 to 100°C. On the other hand, DESs are easily prepared by simply mixing the components (the HBD and HBA) at low temperature (≤ 80°C). The mixture is then cooled down to room temperature and no further purification is needed, with an overall amount of time that could go from 30 minutes to 6 hours and 100% atom efficiency [62]. The self-association between the compounds occurs through the formation of intermolecular hydrogen bonds which, in a defined molar ratio, cause a deep reduction of the melting point, resulting in a liquids-state mixture [63]. DESs, like ILs, are low-volatile, non-flammable, chemically and thermally stable which make them easily storable [27]. DESs are quite high in terms of their viscosity and lower in terms of their conductivity than other ionic liquids and molecular solvents [64]. The polarity, together with the viscosity and density of DESs largely depend on their components, making this class of solvents highly customisable based on the specific application.

DESs, NADESs and THDESs: general classification and constituents

Generally, DESs are classified in four different types based on the nature of the complexing agent used (Table 10).

DES Type	General formula	Description
Type I	$Cat^+X^-zMCl_x$	quaternary ammonium salt and a metal chloride
Type II	$Cat^+X^-zMCl_x \cdot yH2O$	quaternary ammonium salt and a metal chloride hydrate
Type III	Cat^+X^-zRZ	quaternary ammonium salt and a HBD
Type IV	$MCl_x + RZ = MCl_{x-1}^+ \cdot RZ + MCl_{x+1}^-$	metal chloride hydrate and HBD

Table 10. Classification of deep eutectic solvents [64].

A fifth type has been recently introduced and includes only non-ionic HBAs and HBDs, such as the hydrophobic mixture made by thymol and menthol [65-66]. There are several chemicals reported in literature that are employed to form DESs, the most common ones are listed in the following figure (Figure 9) [67]. Moreover, DESs can be made of active pharmaceutical ingredients like ibuprofen, lidocaine, and phenylacetic acid. When at least one of the components is a therapeutic agent, the solvents are named therapeutic deep eutectic solvents (THDESs). They have been developed with the goal of improving the solubility of the active, its membrane transport, drug delivery, and bioavailability [67-68].

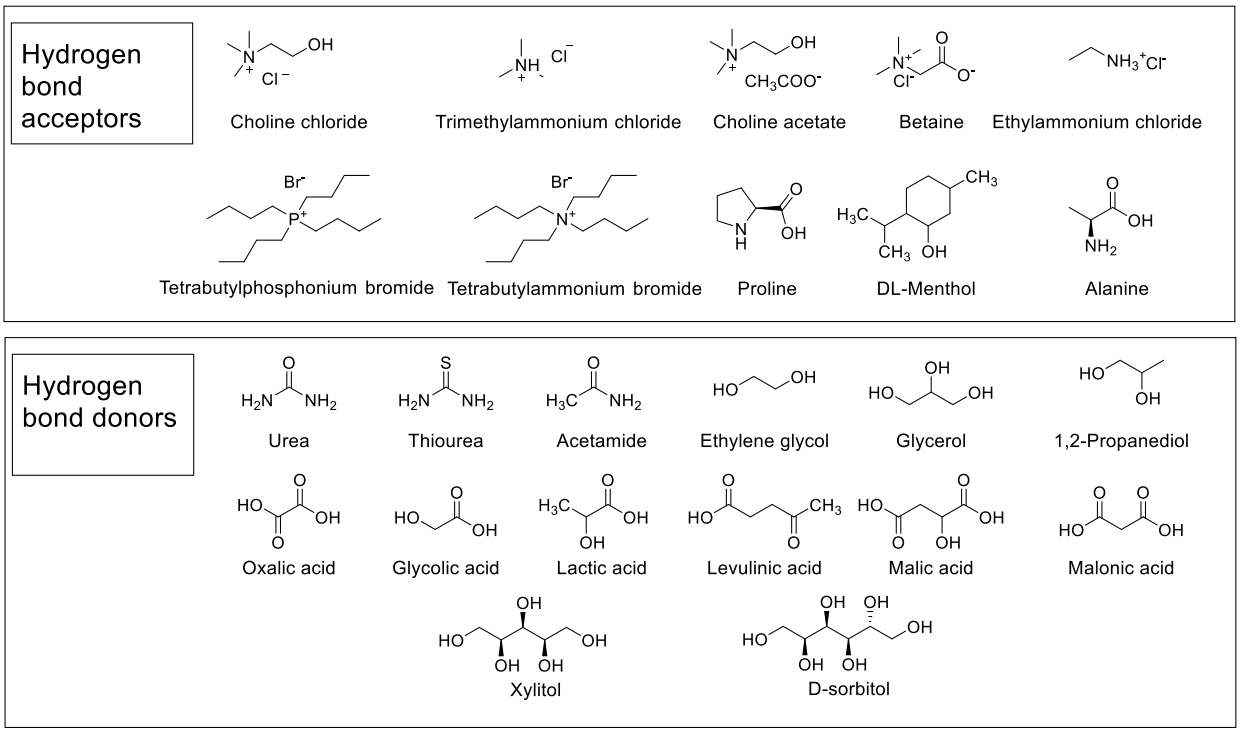


Figure 9 Common components of DESs.

In 2011, Choi and colleagues introduced the definition of "natural deep eutectic solvents" (NADEs), which indicates a DES that is made of primary metabolites such as organic acids, amino acids, sugars, polyols, and choline derivatives [69]. Anyway, is not uncommon for some authors to refer at NADESs simply by the nomenclature of DESs. The fact that their components are natural products made NADESs particularly attractive. They are, in fact, considered "bio compatible" since they can be virtually biosynthesised as well as metabolised by essentially all organisms, helping with potential environmental hazards [70]. Even if NADESs could be considered DESs of type III, a recent classification of NADESs has been proposed and divided in five different classes:

NADESs classification	
1	Ionic liquids, made of an acid and a base
2	Neutral, made of only sugars or sugars and other polyalcohols
3	Neutral with acids, made of sugar/polyalcohols and organic acids
4	Neutral with bases, made of sugar/polyalcohols and organic bases
5	Amino acids-containing natural deep eutectic solvents, made of amino acids and sugars/organic acids

Table 11. NADES' classes [67].

Hydrophobic and Hydrophilic DESs/NADESs

Considering the nature of components (*i.e.*, as sugars, alcohols, acids and some organic salts) NADESs and DESs are usually hydrophilic mixture. However, in the last years interesting mixtures of hydrophobic nature have been reported, composed by hydrophobic compounds such as tetrabutylammonium bromide, menthol, thymol, and fatty acids as HBAs combined with long alkyl chain alcohols and carboxylic acids as HBDs (Figure 10).

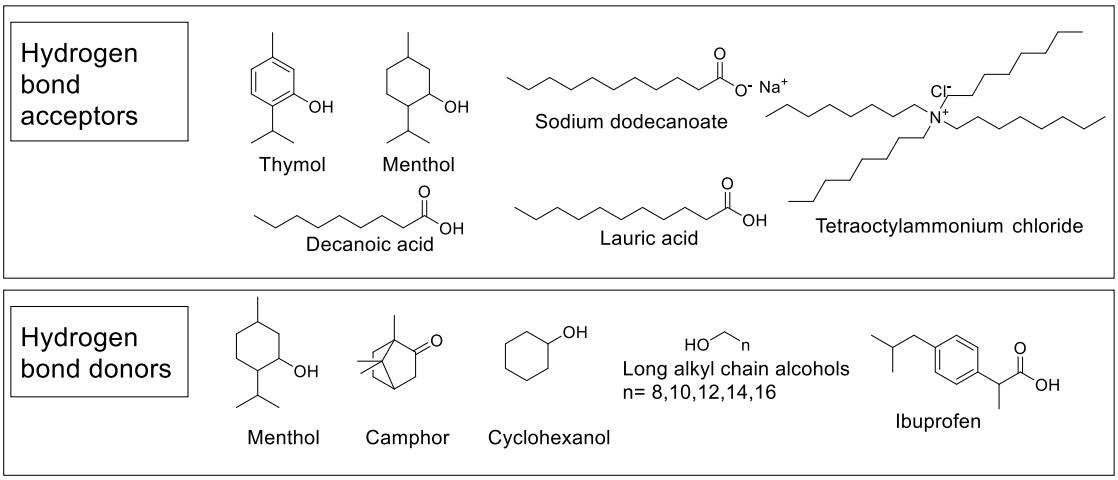


Figure 10. Reported component of hydrophobic DESs, NADESs and THDESs [71].

Comparing hydrophilic and hydrophobic deep eutectic mixtures, it is possible to find two major differences, the second direct consequence of the first. Considering the chemical structure of the parent compounds, we can observe that in the hydrophobic mixtures there is the presence of long alkyl chains while the focus for hydrophilic mixtures is on the hydrophilic domains, such as charges of the salts, and hydrophilic groups, such as carboxylate and hydroxyl group. Consequently, due to the presence of charged and polar moieties there are extensive hydrogen bond interactions in hydrophilic DESs, which lead to a significant depression of the melting point. On the other hand, it was observed the same behaviour in hydrophobic DESs only when a salt is present. When neutral components are present, these mixtures are essentially eutectic mixtures, showing small depressions in the melting points. However, these fluids are still valuable solvents for different applications [71].

DESs and NADESs applications

The applications of this novel class of solvents are widely and constantly evolving. At the beginning, one of the major applications of DESs was in the field of metal processing, for metal electrodeposition, electropolishing, extraction and processing of metal oxides. The advantages of using DESs rather than aqueous electrolytes are the high solubility of metal salts, the absence of water, and high conductivity compared to non-aqueous solvents. But metal finishing applications are not the only ones, in fact DESs have been recently employed in different separation processes and in CO_2 capture [64-65]. Hydrophobic DESs are immiscible with water, feature that makes this class of solvent particularly interesting in extraction and separation processes [71-72]. Also NADESs gained attention for the extraction and separation of various target compounds from natural products.

DESs and NADESs have been employed successfully in biocatalytic processes as reaction media, co-solvents, extractive reagents for enzymatic products, and pre-treatment solvent of enzymatic biomasses. Considering the high tunability of DESs, it is possible to tailor the best eutectic mixture for each enzymatic system. Considering this, DESs allow to:

- improve enzyme activity and stability
- increase reaction yield
- enhance/modify biocatalyst stereo-preference
- contribute to the overall greenness of the process (including recycle and reuse) [63], [72].

DESs are implied also in biomass processing, nanomaterials synthesis, to study the genomic/fundamentals of nucleic acids, in power systems and battery technologies. NADESs could replace organic solvents such as DMSO as dissolving media in biological assays, and together with DESs could act as new vehicle for drug delivery [70].

Biocatalysis

Be an enzyme - a catalyst for change. As a slogan, I don't know if that's ever going to be right up there with Ich Bin Ein Berliner, or "I Have a Dream," but there's a lot of truth to it.

Pierre Omidyar, eBay founder

It is not uncommon in human history that big and crucial scientific discoveries have been made after that something has been exploited for years, or sometimes even centuries, in a complete unaware way. This is the case of microorganisms which had a huge impact on humankind, from not only a social point of view, but also economical. With wars, they were the major responsible of deaths and diseases, fears of the most powerful men that in front of the "invisible" and unknown enemy for centuries did not have any effective weapon. However, at the same time microorganism were employed on a daily basis in a favourable way (without a real awareness on what was going on) for the production of food and beverages: bread leavening, beer brewing, milk curdling, grapes fermentation to vine and vinegar, practiced by humans for at least 6000 years, are all processes mediated by microorganisms. In the course of time, it was discovered that microorganisms could modify certain compounds by simple, chemically well-defined reactions, which were further catalysed by enzymes. Nowadays, these processes are called "biotransformations" [73]. With the time, the advantages brought by enzyme mediated modifications have been employed not only for food's preparation, but also in different fields, like in the production of materials, of pharmaceuticals, fine chemicals, fragrances, and flavours. The interest in biocatalysis has gained more and more attention since huge steps forward have been made in the design and production of enzymes that are now not only able to perform a number of reactions that was not imaginable some years ago, but also available at reasonable prices and in large scale [74]. For these reasons, biocatalysis is not confined anymore in academia, but the industrial interest is becoming stronger every day. Moreover, biotransformations are not just useful to obtain highly productive processes, but they are also favourable for the environment. The use of catalysts is one of the twelve principles of the Green Chemistry, and biotransformations are chemical reactions catalysed by natural compounds, like enzymes, whole cells or microorganism, which are completely biodegradable and *de facto* the perfect ecological option.

Biocatalysis has some unique features. Biocatalytic reactions are *selective*, and selectivity is the primary goal if we want to design a sustainable and a profitable process. Biocatalysts are *substrate selective*, they have the ability to distinguish and act on a subcategory of compounds within a larger group of chemically related compounds. A biocatalyst exerts *functional group selectivity*; it means that an enzyme acts on a single type of functional group. As a result, biocatalytic reactions tend to be cleaner and laborious purifications of products from impurities emerging through side-reactions are not required. Moreover, a biocatalyst is *regio-specific* and *stereo-specific*. The regio-specificity is addressed to the complex three-dimensional structure of enzymes, which can distinguish between functional groups which are situated in different regions of the substrate. Since almost all enzymes are made from L-aminoacids, enzymes are chiral catalysts, and consequently stereo-specific. Any type of chirality present in the substrate molecule is recognised upon the formation of the enzyme-substrate complex. Thus, the two enantiomers of a racemic substrate may react at different rates (kinetic resolution); moreover, a prochiral substrate can be transformed into an optically active product due to a stereospecific interaction with the reaction intermediate. Biocatalysts require mild range of reaction conditions like temperatures (usually between 20-40 °C), atmospheric pressure and pH range (typically 5-8). The generation of side-products due to undesired side-reactions is minimised, raising yields, and facilitating product recovery. Moreover, the usual solvent for biocatalysed reactions is water. Since enzymes could be very efficient, the chemical reactions could be significantly speed up and high reaction rates are achievable [75–77].

Myths about enzymes

In the past, some concerns about the use of enzymes on industrial level have been exposed, but the limits that avoid a wide use of this enabling technology some years ago have been successfully overcome. These are some of the myths revolving around biocatalysis and the answers to them.

"Enzymes are expensive"

Price is always the first concern when industrial scale production is considered. What is a feasible expense for a laboratory could become an unprofitable industrial process. When we speak about enzymes, we cannot just consider the final price to determine the cost impact. Based on the work of Turvfesson and colleagues, we must consider that the actual price of a biocatalyst depends on the economy of scale of production (larger the scale lower the price) and the purity and formulation of the enzyme used (highly purified enzymes will be more expensive but not always necessary to the process) [78]. Moreover, as Rozzell stated back in 1998, we must also evaluate the cost-

contribution of the enzyme to the final product. To support this statement, he brought some examples: for the production of *p*-fluoro-L-phenylalanine, a transaminase cost is $500 per kilogram or more, but its cost-contribution to the final product is only about $20±30 per kilogram, far less than that of the raw materials. The cost contribution for penicillin amidase in the splitting of penicillin G is only about $1 per kilogram, while the cost contribution of aspartase in the production of L-aspartic acid is less than $0.10 per kilogram [77].

"Enzymes are inefficient"

Enzymes are usually very efficient: they are able to accelerate rate-reactions of several orders of magnitude, and usually excel in turnover numbers and frequencies. Furthermore, all the features previously reported (*i.e.*, selectivity, mild reaction conditions) contribute to the obtainment of high-quality products [79].

"Enzymes are unstable"

The stability of an enzyme towards high temperature or extreme pH values has been improved with different approaches: selecting new stable enzymes from peculiar natural habitats, using protein engineering or immobilisation. This last technique limits heat and mass transfer and minimises access of destabilising agents to the enzyme. Moreover, immobilisation onto a surface introduces additional interactions that stabilize the tertiary structure of the enzyme [80].

"Enzymes are highly specific for their natural substrate"

It is true that some enzymes have an amazing selectivity towards their natural substrate, but this is not a truth for all the enzymes. There are also enzymes involved in metabolic or detoxification pathways that excel by a very broad substrate range and high (stereo)specificity making them very useful catalysts [79]. Where a natural enzyme is not available for a specific substrate, the recombinant DNA technology has solved some problems and the number of enzymes is now virtually infinite thanks to techniques as high throughput microbial screening, genomic and metagenomic screening [81].

FOCUS

Enzyme classification

Enzymes are classified in six classes according to the Enzyme Commission appointed by the International Union of Biochemistry and Molecular Biology (IUBMB). The EC classification is universally accepted with a unique name and EC number for each enzyme. The six classes (reported in **Table *12***) are divided on the basis of the general reaction catalysed. Then, each category is further divided into a number of subclasses and sub-subclasses, according to the nature of the reaction catalysed. In this kind of classification, each enzyme is associated with a code of four number (EC X.X.X.X.) with the following meaning:

the first number is one of the six main categories of which the enzyme is related (Table 12)

- the second one is the sub-class
- the third one is the sub-sub class
- the fourth one is the actual number of that enzyme within its sub-subclass

With the formal alphanumeric name is always associated and accepted a trivial name that usually describes the substrate, followed by a reaction, ending by "-ase" [75], [82].

Class	Description
Oxydoreductases	Catalyse oxidation and reduction reactions
Transferases	Catalyse the transfer of functional by means of a nucleophilic substitution reaction
Hydrolases	Catalyse the addition of water to a substrate
Lyases	Catalyse addition and elimination reactions, as cleavage of C-C bonds
Isomerases	Catalyse the rearrangement of atoms within a molecule such as racemization and epimerization
Ligases	Catalyse the formation of bonds between two molecules

Table 12. Classes of enzymes.

Isolated enzymes or whole cells

Biocatalyst could be embodied from isolated enzymes or whole cells. Generally, it is necessary to consider the type of reaction, the cofactor recycling and the biotransformation scale to decide what to employ as catalyst, if whole cells or purified enzymes. In fact, the use of isolated enzymes allows the obtainment of high specific activity and high selectivity, but it is necessary to supply specific cofactors, highly pure preparation could be expensive and there could be problems in the recovery of the biocatalyst. On the other hand, whole cells are one of the cheapest options, easily recoverable and already own cofactors, and systems for their regeneration. The presence of the residual cell wall could be a double-side feature: it is necessary to preserve it for the proper functioning of the system but at the same time is a favourable feature when we work with unconventional media, *i.e.*, not aqueous reaction environment. In fact, usually water is the election solvent for biocatalytic processes, but novel green solvent systems are starting to be used combined with biocatalysis in order to broader the substrate scope, the productivity and also the ecological impact of a synthetic procedure [83]. However, whole cells could show competing enzymatic activities, low specific activity and require facilities for fermentations. Advantages and disadvantages associated with the two different systems are summarised in Table 13.

ISOLATED ENZYMES		
FORM	ADVANTAGES	DISADVANTAGES
Dissolved in water	• Simple apparatus • Simple work-up • Better productivity • High substrate concentration tolerance • High catalytic activity	• Cofactor recycling necessary • Side reaction possible • Lipophilic substrates insoluble • Workup requires extraction
Suspended in organic solvents	• Easy to perform • Simple workup • Lipophilic substrates soluble • Easy enzyme recovery	• Reduced catalytic activity
Immobilised	• Easy enzyme recovery • Enzymatic recycling • Possibility to use continuous flow reactors	• Loss of activity during immobilisation

WHOLE CELLS		
FORM	ADVANTAGES	DISADVANTAGES
Growing cultures or resting cells	• No cofactor recycling necessary • High activity • Simple work up • Fewer by-products	• Expensive equipment • Tedious workup • Low productivity • Low substrate concentration tolerance • Low organic solvents tolerance • Uncontrolled side reactions
Immobilised	• Possible cell re-use Use in continuous flow reactors	• Loss of activity during immobilisation

Table 13. Advantages and disadvantages of whole cells and isolated enzymes.

Immobilisation of the biocatalyst

The biocatalyst form, isolated enzyme or whole cells, is not the only point that has to be set when a biocatalytic process is developed. Once it is defined which is the biocatalyst, it is important to consider if it is more convenient to employ it in its free form or in its immobilised form. When we speak of "immobilisation" we are talking of fixing, making the biocatalyst "immobile" on a support. The definition of "immobilised biocatalyst" was proposed at the first Enzyme Engineering Conference that was held at Henniker, NH, USA, in 1971, as "biocatalysts, enzymes, or cells are physically fixed in a defined region in order to catalyse a specific reaction with no loss of catalytic activity and with repeated use" [84]. As already mentioned, costs and stability of the biocatalyst are two major concerns related to biocatalysis on industrial level, and immobilisation could be the answer to both problems. In Table 14, some large-scale processes using multi ton to multi-thousand ton per year of immobilised biocatalyst are reported.

Enzyme	Industry	Type	Process
Nitrile hydratase	Chemical	IWC	Acrylonitrile hydrolysis to acrylamide
Penicillin amidase	Pharma	IME	Manufacture of β-lactam antibiotics
Glucose isomerase	Food	IWC/IME	High-fructose corn syrup
Aminoacylase	Chemical	IME	D- and L- aminoacids
Lipase	Food	IME	Cocoa butter analogues
Lipase	Food	IME	Omega-3 ethyl esters
Epimerase	Food	IME	Allulose sweetener
Lipase	Chemical	IME	Enantiomerically pure amines
Lipase	Chemical	IME	Enantiomerically pure herbicides
Transaminase	Pharma	IME	Sitagliptin

IWC = immobilised whole cells; **IME**= immobilised enzymes

Table 14. Large scale productions employing immobilised enzymes or whole cells [85].

Immobilisation of enzymes

Soluble enzymes are, by definition, dissolved into the reaction media and the separation at the end of the process from the product could be a complicated and expensive procedure. On the other hand, the use of an immobilised enzyme, which is confined on a solid phase, allows the easy and simple recovery through filtration or centrifugation and its recycling, permitting to be reused several times. Since immobilised biocatalysts are easily separated from the reaction mixture, the downstream process and the purification steps are easier and less expensive. The immobilisation improves the activity, the selectivity and most of all the stability of the enzyme providing longer half-lives and less degradation. Immobilisation, in fact, prevents the unfolding of the tertiary structure of the enzyme improving its stability and allowing the biocatalyst to be used under a wider range of reaction conditions, including water immiscible organic solvents. Moreover, immobilised enzymes become suitable for continuous application, but I will discuss later this application. Another advantage related to the confining of the biocatalyst is the prevention of the protein contamination of the final product and the avoiding of phenomena of product inhibition. Furthermore, since an immobilised enzyme cannot easily penetrate biological membranes, it has low or no allergenicity [84], [86].

Immobilisation of cells

Some of the considerations made for the immobilisation of enzymes are still valid for cells' immobilisation. Anyway, there are some differences in the nature of the two biocatalysts that made necessary some clarifications. Since immobilised cells could be used repeatedly and continuously,

the cost of bioprocess is consequently reduced thanks to the maintenance of high cell density during process. Immobilised cells are a more robust system, due to the improved resistance to shear stress given by the presence of an additional support material that provides protection, high resistance to abrasion, non-compressible particles and low leakage of cells. Compared to enzyme immobilisation, cells immobilisation brings some advantages like less expense on separation, advantageous isolation, and purification method, allows multistep reactions and continued biosynthesis by cells [84].

Limitations of immobilisation

Since everything has a double-side, immobilisation is not the exception. Limitations related to the lock of the biocatalyst are related both to the nature of the biocatalyst itself as well to the nature of the support. The cost of the carrier is an additional expense that could impact on the total cost of the procedure. The fragility or robustness of the immobilisation support must be considered since abrasion of the solid particles by mechanical stirring could result in loss of activity of the biocatalyst. Moreover, a decrease in the enzymatic activity could be the result of a wrong conformation given by the interaction between the enzyme and the carrier. Because it is a heterogeneous catalyst, activity loss can also result from diffusion limitations, in particular with macromolecular substrates, and dependent on the particle size [85]. For immobilised cells in this regard is crucial to choose a matrix that allows to have at the same time a high cell density and a good diffusion between the caged cells and the culture media. Other disadvantages related to the use of immobilised cells depend on the area of application. Eş and colleagues reported that "*the cells to be immobilised might have different activity and be in different growth phase to avoid their unrestrained growth, which can burst the thin layer of support matrix used for mostly entrapment of organism and this might contaminate the product in production media*" [84].

Immobilisation methods

Generally speaking, the immobilisation techniques can be divided in three categories: binding to a support (carrier), entrapment (encapsulation) and cross-linking.

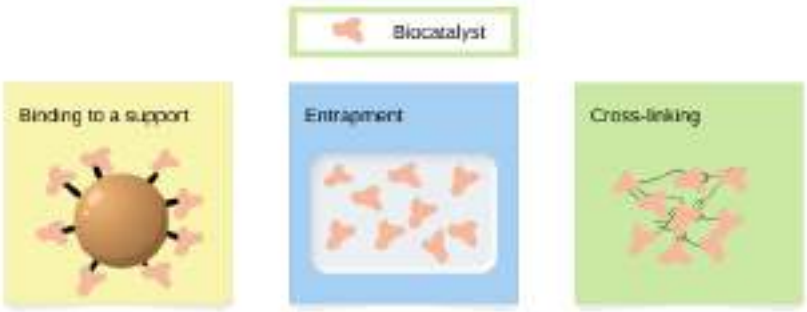


Figure 11. Different type of immobilisation.

Binding to a support

The immobilisation through binding to a solid support is a technique which could occur through physical adsorption, ionic binding, or covalent binding. Physical adsorption is a type of immobilisation which requires no permanent bonds, but only weak bonds such as ionic interactions, hydrogen bonds, Van der Waals forces. Its advantage is the lack of pore diffusion limitation, anyway its efficiency is low, and the binding is too weak to allow the use of the immobilised biocatalyst under industrial conditions of high reagent and product concentration and high ionic strength. Covalent binding involves the formation of covalent bonding between a reactive group of the enzyme and a reactive group of the support or carrier. This kind of binding to a carrier is robust and strong enough to be largely employed. However, the limitation of covalent binding lays in the impossibility to recover both the biocatalyst and the support in the case of the irreversible inactivation of the enzyme [87]. Furthermore, when a biocatalyst is bonded to a solid support a dilution of catalytic activity is observed, since a large amount of non-catalytic mass (90-99% of the total) is added. Consequently, there is a lower volumetric and space-time yield, together with a lower catalyst productivity [88].

Entrapment

Entrapment is considered the primary immobilisation method for whole cells, resting or viable [85]. It consists of physical immobilisation of biocatalyst in a porous matrix of different nature typically organic or inorganic polymer matrices, or membrane devices such as a hollow fibre or a microcapsule [87]. It can have covalent or not-covalent bonds. It is a fast method, but it has pore diffusion limitation. The difference between entrapment and binding consists in the support: binding when the support is preformed, regardless to the position of the biocatalyst (on the surface or inside the pores of the support), whereas entrapment when the matrix is formed during the immobilisation process [84], [87].

Cross-linking

In a cross-linked enzyme aggregate (CLEA), enzymes are directly linked by covalent bonds, there is not support or matrix, which makes this method simple and cheap. It consists in two easy steps: precipitation and cross-linking. The precipitation of the enzyme from an aqueous solution is obtained thanks to the addition of a precipitating agent, *i.e.*, salts, or water-miscible organic solvents or non-ionic polymers. Proteins precipitate as physical aggregates, held together by non-covalent bonding without perturbation of their tertiary structure. The cross-linking of the resulting aggregates is performed using bifunctional cross-linking chemical groups, such as glutaraldehyde [88]. The advantages of cross-linking techniques lay in the high concentration of enzyme activity in the final macroparticle, the high stability and low production costs associated with the procedure that do not require an additional solid support. However, there is the risk that enzymes can lose their activity because of covalent bond between them during the cross-linking phase [84], [87].

Immobilisation supports

The ideal support for immobilisation should have the following features:

- Minimum cost-contribution, *i.e.*, enzyme costs are <5%.
- Sufficient availability and environmental compatibility.
- Good physical and mechanical stability that allow the use in different reactor, configurations, and solvent systems.
- Large surface area and good porosity which enable an efficient mass transfer and high activity.
- Optimum affinity for the biocatalyst considered in terms of hydrophobicity/hydrophilicity.
- A functionalised surface.
- Facile recovery and recycling [85].

The choice of the most suitable support material results to be a crucial step. There are numerous supports for immobilisation, including both organic and inorganic materials, and novel supports are continuously designed to improve the field of biocatalyst immobilisation.

Agar and agarose

Agar is a polysaccharide extracted from the cell walls of red seaweeds, belonging to *Rhodophyceae* class, widespread all along the world (including Japan, Korea, Spain, Portugal, some African countries, Mexico, Chile, and India). Agarose is a linear polymer derived from the purification of

agar with gel-forming properties. Agarose-based beads are highly porous, mechanically resistant, chemically and physically inert, and sharply hydrophilic. Agarose gel contains pentagonal pores with proportional size to allow diffusional access of many proteins. On the other hand, agarose gel does not have a strong mechanical structure [84], [89].

Collagen

Collagen is a polymer largely used because of its biocompatibility and ability to attach cells. It is the main protein of the connective tissues of the animal bodies. In fact, tendon, skin, bone, and cartilage are sources for its isolation and this made collagen naturally available. However, the main issue is the expensive purification process [84].

Chitosan

Chitosan is a polysaccharide obtained by deacetylation of chitin, a structural component found in crustacean, shells, fungi, insects, and molluscs. Chitosan can form hydrogels by ionic or chemical crosslinking with glutaraldehyde and can be hydrolysed enzymatically. On the other hand, chitosan has weak mechanical properties compared to other support materials [84].

K-Carrageenan

It could be used as an alternative to alginate, thanks to its stronger resistance to chelating agents. Anyway, beads made by this polysaccharide could be fragile and leak immobilised material into the medium. The solution to this problem could be the incorporation of small amounts of locust bean gum to reinforce the beads. K-Carrageenan rigidity is related to high potassium concentration [84].

Alginate

Alginic acid is an unbranched binary copolymer of 1-4 glycosidically linked α-L-guluronic acid and its C-5 epimer β-D-mannuronic acid. Alginate are named the salts (and esters) of these polysaccharides. Alginates are generally extracted from seaweed like marine. The proportion and distribution of α-L-guluronic acid and β-D-mannuronic acid (which depends on the origin of the alginate) is related to the physicochemical properties of this polymer, like the different internal pore size of the gel beads, which then determine the rigidity of the sphere. The high biocompatibility, low toxicity, hydrophilicity, and simple gelation made alginates the preferred material for whole cells and enzyme immobilisation. Alginate forms gels with most di- and multi-valent cations. Monovalent cations and Mg^{2+} ions do not induce gelation, whereas ions like Ba^{2+} and Sr^{2+} produce stronger alginate gels than Ca^{2+}. The gelation process takes place thanks to the ionic reticulation between the negatively charged carboxylic groups of alginic acid and the divalent ions. The main

issue with alginate beads is the loss of divalent ions into surrounding fluids, which causes the uncontrollable and unexpected dissolution of the matrix. The immobilisation of enzymes or whole cells into alginate beads is a method usually divided into two phases: firstly, a dispersion of the biocatalyst into a solution of alginic acid is prepared, then the dispersion is solidified thanks to the interactions with divalent ions and the gel formation (*e.g.*, dropping the dispersion into a cold stirred solution of $CaCl_2$) [84], [90-91].

Graphene oxide

Graphene oxide is the precursor of graphene in its large-scale synthesis from graphite and is considered an ideal candidate for immobilising enzymes. It combines high surface area and great thermal and mechanical properties together with plenty surface functional groups (carbonyl, carboxyl, hydroxyl and epoxy) that give it with good dispersibility in water [85].

Polyvinyl alcohol

Polyvinyl alcohol (PVA) is a synthetic polymer largely used for immobilisation since is safe for microorganisms and an easy and cheap method at industrial scale. Furthermore, PVA beads have elasticity and high mechanical strength that are theoretically adequate for high shear stresses [92]. Usually, the immobilisation of the biocatalyst is performed mixing the biocatalyst with a solution of PVA and the gelation is promoted by boric acid or sodium sulphate, which induce the cross-link between PVA molecules [93].

Nanomaterials

Nanoscale materials as nanotubes, nanofibrous membrane, nanoparticles, and mesoporous materials, have been exploited as immobilisation carriers. Advantages of this kind of supports are related to the high surface area, the limited diffusional problems since all enzyme molecules are located on the surface of the particle of non-porous nanomaterials, the effective enzyme loading, and the increased stability given by multipoint binding to non-porous materials. On the other hand, nanomaterials could have significant cost of production, which could prevent large scale applications, and separation from reaction media could be difficult. Different methods of immobilisation have been exploited with nanomaterials, from entrapment to covalent binding [94].

Silica

Silica (SiO_2) is another material suitable for enzyme immobilisation; it could be classified based on its physiochemical and morphological characteristics. Silica surfaces could be functionalised with different compound, like PVA and polyethylenglycol (PEG), together with many others.

Mesoporous silica shows some advantages as a carrier for biocatalyst immobilisation, thanks to the uniformity of its pore diameters (2-40 nm), the high surface areas (300-1500 m^2g^{-1}) and the pore volumes (around 1 mL g^{-1}). Moreover, it is inert and stable at high temperatures [94].

Traditional and unconventional media for biocatalysis

In the previous paragraphs, I deeply discuss the ecological impact of solvents, which are the parameters to consider assessing the safety and environmental hazards of solvents and the technological development in order to design and produce novel systems that are able to overcome these limitations maintaining the intact the primary function of solvents. In this context, it is interesting to describe how this topic is related to biocatalysis and which are the common and less conventional media usually employed for biotransformations.

Water

For years scientists tried to replace organic solvent with water, which appears to be the best options to adopt (when possible) due to its intrinsic features like safety, low-cost, non-flammability, and availability. Water in its liquid form is a polar protic solvent, while in its supercritical form acts as a non-polar solvent due to the loss of hydrogen bonds and exists at pressures above 221 bar and temperatures above 374 °C. Even if examples of synthetic procedure performed in aqueous system, pure or mixed with other solvents, are reported in literature, organic chemistry in water remains rare [27], [95]. However, a separate topic appears to be biocatalysis which not only allows to perform chemical reactions with high selectivity but also recognises water as the solvent of election. Life depends on water, living cells contain around 75% of water, most enzymes and many essential cofactors are purely soluble in water-based solvents. Buffer solutions are the most used reaction media for biotransformations when reactants are soluble enough to be dissolved in water. Otherwise, the addition of hydrophilic water-miscible compounds can increase their solubility (co-solvents). Organic solvents, ionic liquids, deep eutectic solvents or surfactants could be used for this role, and, in some cases, also the co-substrate can act as dual-function solvent. Since the environment remains monophasic, there are no limitations due to mass transfer or deactivation at the surface of the biocatalyst. However, the presence of these additives could alter properties like stability, activity and even chemo-/stereo-selectivity or substrate specificity [96–98].

Biphasic solvent systems

When the addition of a co-solvent is not enough for the solubilisation of the substrates, or in case of higher concentration, a biphasic system usually made by water and a water immiscible phase (*i.e.*,

organic solvent, ionic liquids, deep eutectic solvents) could be the best option. In biphasic systems, usually the aqueous phase contains the biocatalyst and hydrophilic cofactors, whereas the water immiscible solvent contains the hydrophobic substrates and the products. In case of free biocatalyst, the mass transfer of reagents between the aqueous and immiscible phase is easily achieved by shaking or stirring. When immobilised enzymes and cells are used, the situation is more complex, and the penetration of reagents into cells or matrices may be rate limiting. Moreover, it must be taken into account the possible deactivation of the biocatalyst on the interface [97], [99].

Non-aqueous system

The complete replacement of the aqueous environment could seem counterintuitive talking about biocatalysis but is a well-established reality. The stability of a biocatalyst inside a non-aqueous media relies on different conditions, starting from the nature of the biocatalyst, from its form (free or immobilised), the presence or absence of additives (*i.e.*, sugars), the water content and the type of non-aqueous solvent. No general rules are available, but literature reports several cases of biocatalyst employed in this kind of media with interesting advantages:

- Catalysis of chemical reactions that are not feasible in aqueous media, for example, unfavourable thermodynamic equilibrium in water.
- Easy work-up procedure (separation of biocatalyst and organic phase containing the product).
- Higher solubility of hydrophobic substrates in hydrophobic non-aqueous solvents.
- Suppression of water-dependent side reactions.
- Improvement and control of chemo-, regio-, and stereo-selectivity.
- Recovery and reusability of enzyme even without immobilisation.
- Alteration of the partition of substrates/products: aids separations, improve yields, elimination of substrate, and product inhibition of biocatalysts.
- Enhancement of thermostability of the biocatalyst in nearly anhydrous organic solvent system.
- Elimination of microbial contamination.

Limitations associated with this kind of reaction media are related to the possible inactivation of the biocatalyst, mass-transfer limitations in the case of viscous solvents and the need of water activity control for processes involving condensation reactions [100]. Solvents used as the media of

biotransformations range from classic organic solvents (*i.e.*, ethyl acetate) and bio-based solvents (*i.e.*, 2-MeTHF and Cyrene ^{TM}), to ionic liquids and deep eutectic solvents [101-102].

Neat substrate system

Even if few literature examples have been reported, neat substrate systems deserve few words. In these systems, the solvent is completely absent, and the biocatalyst is dissolved directly into the substrate (with the addition of small amount of water if necessary), allowing to obtain the highest concentration of substrate possible, which can lead to impressive productivities. However, there is also the possibility that the biocatalyst will show a decrease in stability, activity and selectivity under these conditions [97].

Flow chemistry

"The only way to make sense out of change is to plunge into it,

move with it, and join the dance"

Alan Wilson Watts, philosopher

Chemistry is not a recent discipline and despite the stunning advancements made by scientific progress there is an aspect that in chemistry has remained untouched for centuries: the laboratory's equipment. The standardised glassware has not been changed for over a century, but, even before, the differences between the alchemist's tools and the modern chemist's equipment were not so dramatic [103]. Figure 12 is a sixteenth century representation of a metallurgical workshop, where are clearly visible instrument that are familiar to contemporary organic chemists, like the separating funnels on the table [104].

Figure 12. Hans Weiditz, "The Alchemist" 1520.

On the other hand, Figure 13 is an illustration which shows the laboratory of Professor Justus Freiherr von Liebig, opened in 1824 and one of the first university-level laboratory to which students were allowed. As stated by Liebig "*At that time, chemical laboratories in which instruction was given in analysis did not exist anywhere; what people called such, were rather kitchens, filled with all sorts of furnaces and utensils for carrying out metallurgical or pharmaceutical processes*." The Professor developed not only a teaching method that alarmingly remembers the teaching method of actual graduate-level chemical research program, but also the instrumentation inside the

laboratory that appears to be just an older version of what plenty educational academic (and not) laboratories currently possess [105].

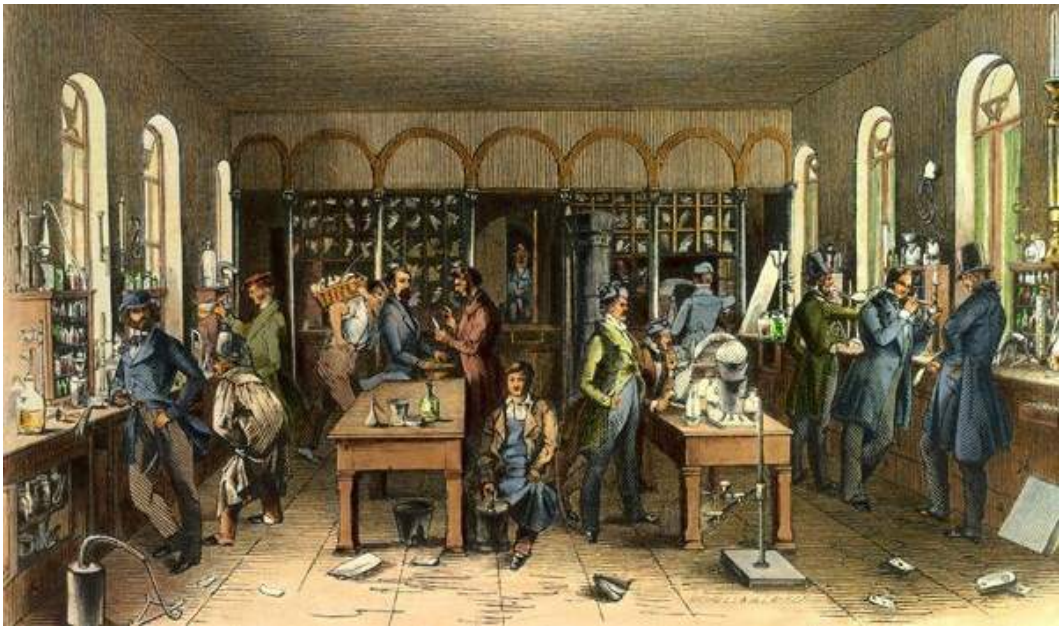


Figure 13. Liebig's laboratory (1803-1873), by Wilhelm Trautschold 1912.

Tools such as standard glass round bottom flasks, condensers, measuring cylinders, test tubes and Bunsen burners are all still commonly in use today, despite them being invented over 160 years ago. A classical synthetic process carried out in a laboratory use a typical batch set-up composed by a vessel where the reaction takes place, a work-up/quenching step which usually employs glassware that allow separation and filtration procedures. Then, there is the removal of the solvent (*i.e.*, evaporation), followed by purification steps (*i.e.*, chromatographic column, distillation, crystallisation). A standard sequence for a reaction today and over a century ago would still be easily recognisable to a modern chemist and chemist directly arrived from the nineteenth century [106].

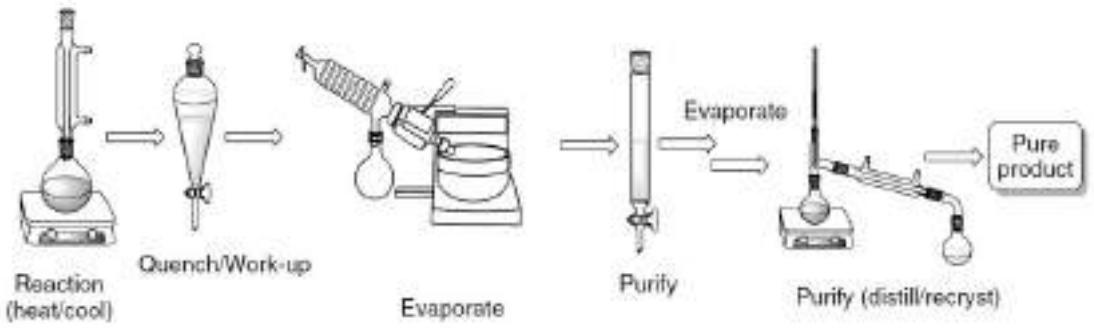


Figure 14. Batch standard workflow [106].

Batch synthesis is a well-established method but there are limitations associated to it, like poor reactivity, low selectivity, incomplete reaction, or extensive by-product formation, which is often a result of poor mixing and temperature control in conjunction with the use of highly reactive reagents [106]. Flow chemistry has been able to successfully overcome all these problems giving an answer to the long-waited need of modernization inside organic chemistry laboratories as well as chemicals manufacturers. The basic principle of flow chemistry is to perform reaction in a continuous manner thanks to the use of channels and tube combined to different pumping systems, rather than in traditional flasks. The system is directly connected to a quenching unit, to pressure regulation devices, optionally to an in-line purification system and analysis devices, ending with a collection system. (Figure 15).

Figure 15. General set-up for a continuous system [107]. Commercially available flow chemistry systems. (a) Uniqsis FlowSyn reactor (top left); (b) Advion Nanotec reactor (top second); (c) LabTRIX system (top third); (d) X-Cube (middle left) (e) AFRICA flow system (middle centre); (f) Vapourtec R2+R4 system (bottom left); (g) FutureChemistry system (bottom centre); (h) Microreactor Explorer kit (bottom right) [108].

The anatomy of a continuous set-up

Pumps for liquid handling and mass-flow controllers

Nowadays numerous mechanical pumps for fluid transportation exist and could be classified in three main groups based on the method used to move the fluid: direct lift, displacement, or gravity. Pumps that have been generally considered and employed for continuous flow experiments in this doctoral work are here described.

- Syringe pumps: working on the principle of reciprocating positive displacement of a fluid are usually made of a cylinder of different materials and a moving plunger. There are single syringe pumps and more sophisticated multiple syringes pumps where the syringes are working synchronously (while a syringe is pushing the reactants into the reaction zone the other is collecting fluid from the reservoir).
- Peristaltic pumps: are rotary pumps, the fluid is processed inside a flexible tube which is pressed by a rotating mechanism so that the fluid is brought to motion. Thanks to this mechanism, peristaltic pumps are able to pump well-suspended slurries.
- HPLC pumps: HPLC pumps (single and dual piston reciprocating) are commonly used for low to high-pressure applications at flow rates higher than 0.1 mL/min. Since the fluid is in direct contact with the pumping mechanism issues may arise due to fouling and blocking via precipitation with some reagents.

In case of a gaseous reagent the device needed to insert it into the continuous reactor is a mass-flow controller which allows to regulate and control the amount of gas entering the reactor per unit of time [107], [109].

Reactor types

A “reactor” is the place where the reaction takes place. Usually, reactors are classified in three main groups: coil reactors, chips and packed-bed reactors. The nature of the reaction determines the type of the reactor and the material. Regardless of the reactor type, the temperature can be easily controlled by a thermostatic unit, but besides heating and cooling the reactor devices could be subjected to different physical interactions such as sonication, UV light, microwave radiations, in order to promote the reaction. The high surface-to-volume ratios of chip-based reactors guarantee the best heat transfer characteristics. This makes chip reactors the ideal for thermal reactions. The main problem with this type of reactor is the tendency to clog. Coil reactors represent a cheaper alternative to chip reactors. This type of reactor is usually made out of fluoropolymers (PTFE, PFA,

and FEP) or stainless-steel pipes, with diameters of 1/8'' or 1/16''. FEP has the best transmission properties for UV/vis irradiation, while stainless steel is excellent when high-temperature and pressure are required. Packed bed reactors are mainly used when heterogeneous reagents or catalysts are involved. This type of reactor consists of a column made from glass, polymeric materials, or stainless steel, filled with a volume of solid material, with a filter frit at both endings [107], [109].

Figure 16. From left to right: a chip reactor, a coil reactor and a packed-bed reactor-

Reactor size

Flow chemistry devices could be described as micro or mini (meso) reactors depending on the dimension of the internal diameter. The features of these types of reactors are summarised in Table 15.

	Advantages	**Disadvantages**
Microfluidic reactor (i.d. 10-500 µm) Volume in µL range	High heat transfer surface to product volume ratios Good heat transfer capabilities Ideally suited for optimising reaction conditions Efficient mixing	Microchannels suffer from restricted flow capacity High pressure drop Clogging issue
Mini/meso fluidic reactor (i.d. 500 µm – several mm) Volume in mL range	Improved flow capacities Lower pressure drop Less clogging issues Preparation of multigram to multikilogram quantities Possibilities to work with packed bed reactor	Lower heat transfer surface Poorer heat transfer capabilities

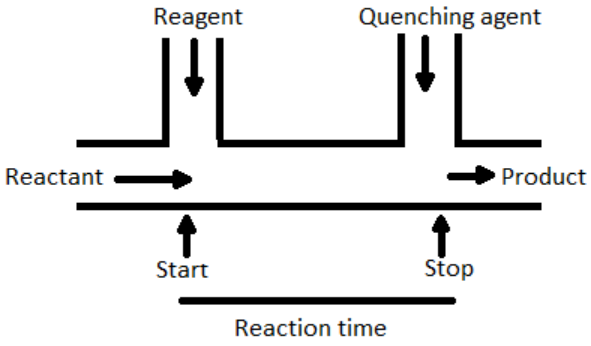
Table 15. Advantages and disadvantages of micro and mini(meso) flow reactor [110], [111].

The major advantage of microfluidic reactors is the high heat transfer, due to the high ratio surface/volume. The scale of micro flow reactors makes them ideal tools for process development experiments. Microreactors are characterised by laminar flow and mixing is limited to diffusion phenomena [111]. On the other hand, they are subjected to high pressure drops that could limit flow capacity and block the whole system. Moreover, the amount of final product collected could be relatively small, even when parallelisation and scale-up are performed, whereas mini and meso reactors could synthesize approximately from 10 mg to an annual production rate of several tens of tons of material of a desired compound [110]. The flow which characterised mesoreactors exceeding 1 mm is turbulent, especially at high flow rates. Between 500 μm and 1 mm i.d. the type of flow generated depends on the flow conditions [111].

Time and concentration

Reaction time in a batch approach is determined by how long reactants are mixed. It usually starts when components are combined at the appropriate activating conditions and finishes when is achieved the best conversion with less side-products formation by adding a quenching reagent to terminate the reaction. In case of very fast reaction, it is difficult to perform the batch approach: for example, if the reaction is complete after 0.5 second and a significant amount of side products is produced due to overreaction after 1 second it is particularly complicated to adjust the reaction time using batch chemistry. Using a flow reactor, the focus is residence time, *i.e.*, the time the reactants stay in the reactor zone, determined by the volume of the reactor and the flow rate. The reactor zone is the space between the inlet position of the reagent and the inlet position of the quencher and can be controlled by adjusting the relative position of these inlets [109].

$$Residence\ time\ (\text{min}) = \frac{Reactor\ volume\ (mL)}{Flow\ rate\ (\frac{ml}{\text{min}})}$$



Scheme 5. Reaction time in a flow reactor and how to calculate residence time.

In traditional batch chemistry, the reaction takes place in a flask, and the concentration of reactant decreases with the proceeding of the reaction, while the product's concentration increases. Consequently, the concentration of reactants and products is uniform at a defined time inside the reaction's vessel. However, in a flow reactor the concentration of the reagents decreases moving through the reactor, while the concentration of the product increases: reagents' concentration reaches the maximum at the beginning of the reactor and then decrease with the distance, opposite behaviour is shown by products. This means that the concentrations of the different species do not change with the progress of operation time under a steady state in a particular position. Therefore, the reaction time in a flow reactor can correlate to the space position inside the reactor and could be controlled by adjusting the length of the reactor itself and the flow rate [109].

	Batch	**Flow**
Stoichiometry	Concentration/ratio of the molar quantities	Concentration/ratio of the flow rates
Reaction time	Time spent under defined conditions	Residence time spent in the reaction zone (defined by reactor volume and flow rate)
Reaction progress	Time spent in a flask	Distance travelled in the channel
Steady state characteristics	It has a uniform concentration at each position within the flask at a particular moment	It has a steady state but different concentration at each position throughout the length of the reactor

Table 16. Comparison between the major reaction characteristics in batch and flow.

Mixing efficiency and mass transfer

Mixing efficiency is a crucial point in the set-up of a reaction. It is defined as the phenomenon that creates homogeneity of all species in the solution. Usually in batch reactions stirring is obtained thanks to magnetic stirring and the limitation step is the molecular diffusion, of which the time is proportional to the square of the length of the diffusion path. Therefore, the diffusion time depends on the size of eddies of solution **B** dissolved in solution **A**. The mixing can be speed up with the decrease of eddy size that is obtained increasing the intensity of turbulence of stirring to get faster mixing. In batch, mass transfer is made by convection, which results in chaotic and turbulence mixing [109]. Only a minimal portion of materials is indeed efficiently mixed. These idle portions lead to poor heat-transfer, concentrate reagents, and to the formation of "heat spots"; so, in this way, reactions are often inefficient. In flow, microchannels have a volume/surface ratio of about 30.000 m^2m^{-3}. This peculiarity and the continuous flow allow complete and homogeneous mixing, that is achieved in terms of microsecond in microreactors [112]. Flow reactors have shorter diffusion time: a molecule in the centre of the channel could reach the wall of a microfluidic channel in few

second. In flow chemistry, two different types of mixing can be identified: active and passive. Active mixing when an external energy input is applied to promote the mixing, like ultrasonication. Passive mixing, on the other hand, occurs at a rate proportional to the fluid properties, pumping speed, and physical path through the respective mixing unit. T-shaped or Y-shaped connection units are the most typical mixing devices usually employed for relatively slow reactions which are not improved by faster mixing.

Figure 17. T and Y mixer units.

Faster reactions require more efficient type of mixing units like static mixers, specialised mixers with optimised microstructures using obstacles within the microstructures or multilamination mixers [107].

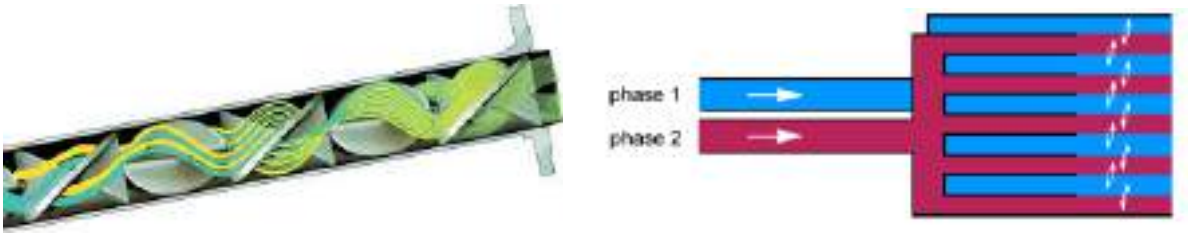


Figure 18. Static mixer (on the left). Multilamination-type micromixers (on the right): flow stream is divided in smaller streams increasing the contact interface between the solution enhancing diffusive mixing which takes place in short time thanks to the short diffusion path.

Temperature control

Heat transfer in flow systems is better than in batch: in fact, the small dimensions of the channels determine a high contact surface for heat exchange. The result is that reagents are homogeneously heated, and the temperature can be precisely controlled. This is a very important issue because a non-homogeneous heating, as in batch systems, can cause the formation of secondary products or reagent and product degradation. Also, the high surface/volume ratio allows to perform exothermic reactions, that are very hazardous in batch, in a safe and controlled way. The increased safety is an important advance of flow chemistry that can have an important impact in industry. The safer

conditions depend on the fact that the used volumes are smaller than ones used in batch conditions, and this allows to better control the reactions and to use also very reactive and hazardous reagents and solvents [110].

Quenching

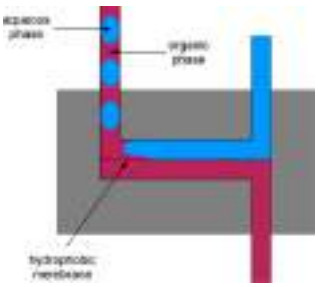
An appropriate quenching strategy is required to get a precise control of the reaction time, otherwise reactions (or side reactions) may continue in the collection flask. Quenching is not necessary with packed bed catalysis and with electro- and photochemical reactions since the reaction stops as soon as the reaction mixture leaves the reactor. For other homogenous reactions, the reaction time is controlled either by chemical or thermal quenching [107].

Pressure regulation

Back pressure regulators (BPR) are valves that maintain a defined upstream pressure. Operating at high pressures allows to perform reactions above the boiling point of reagents and solvents. Pressure sensors are often equipped into the pumping unit [107].

Analysis and purification

Analysis of the reaction mixture can be conducted in two different ways: offline or online. Offline analysis is most commonly done for laboratory scale reactions: reaction mixtures are manually picked and analysed. Instead, online analysis happens through the automatic and periodic transfer of samples of the reaction mixture, which are then transferred to the analytical instrument, like HPLC, GC, MS, IR, NMR. Online analysis is useful for the extensive optimisation of parameters and quality control of the continuous process. The most common purification method used in flow chemistry is liquid/liquid extraction. First, the extraction solvent is delivered to the reaction mixture through a mixing device, and then the biphasic system enters the membrane separation unit, which is a PTFE membrane located between two flow channels. The organic phase can cross the hydrophobic membrane: this is how the two phases are separated.



Scheme 6. Scheme of working principle of a liquid/liquid separator [107].

Another common method of purification is the use of scavenger cartridges. They are packed bed reactors, usually filled with an appropriate resin (*i.e.*, acidic, basic, supported reagents). When installed at proper positions, they are able to remove reagents in excess or impurities [107].

FOCUS

Flow chemistry and the Green challenge

The general overview of the basic continuous toolkit and how reaction parameters change inside a continuous set-up highlighted some advantages that covered not only the chemistry itself and the associated hazard but are also intriguing for industrial development and applications of flow chemistry (**Table *17***). In fact, as already mentioned, from a technological point of view, continuous equipment frequently enables much better control of heat and mass transfer inside the equipment. Therefore, existing processes can be run at higher temperature, pressure, and/or concentration, if advantageous for the reaction, or completely new synthetic pathways become possible that could not be safely carried out in conventional equipment (often referred to as "novel process windows").

Logistics/quality	Chemistry/process	Safety
Increased throughput	More efficient heating and cooling	Improved reaction control for exothermic reactions
Accelerated implementation (shorter lead times, facilitated scale-up)	Novel process windows	Smaller equipment/reduced hold-ups
Reduction of lot-to-lot variations	Direct processing of unstable intermediates/ products	Operation without vapor space
Minimization of inventory and establishment/ validation stocks	Implementation of demanding separations (*e.g.*, multistage extraction)	Chance to use disposable equipment for highly potent or cytotoxic reagents/ products

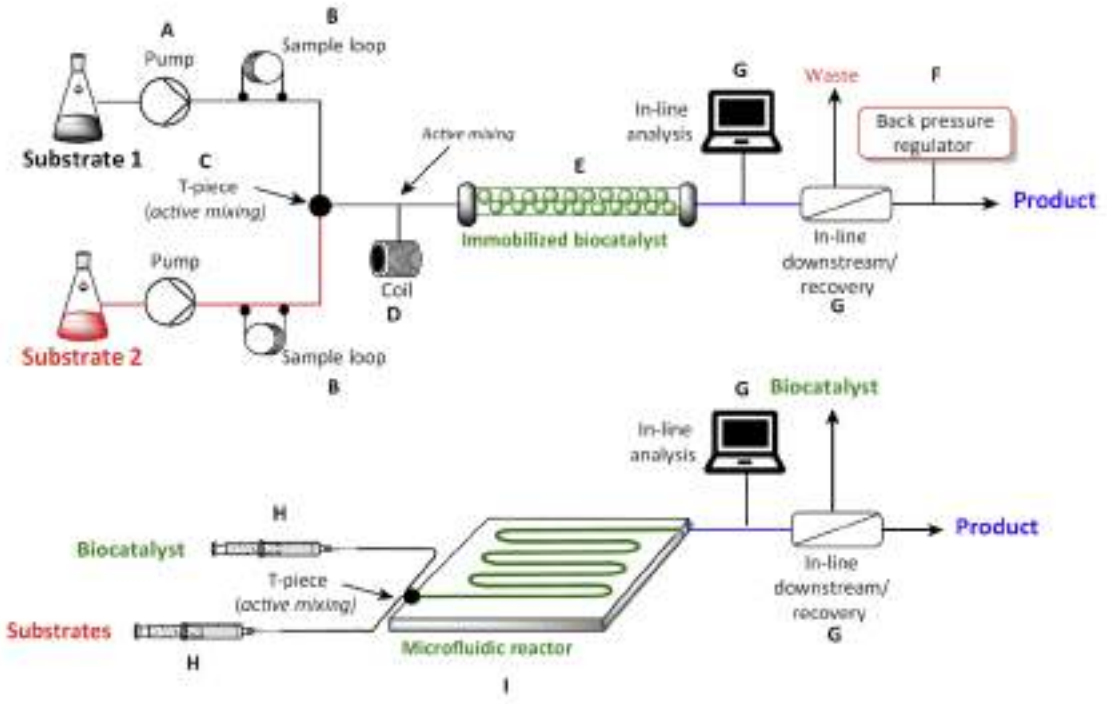
Table 17. Advantages associated to flow chemistry and continuous process.

Flow chemistry has much to offer to pharmaceutical industry, for the easy scale up, easy optimisation and process intensification. Most of all, a peculiarity is the ability of producing large quantities of product in a short time, and this is possible when the synthetic pathway is clean, simple, efficient, safe, reliable, and reproducible. There is a continuous balance between speed and optimisation, and flow reactor technology has all the qualities to be inserted in these transitions. Moreover, there is also the improved ecological impact that a continuous process could have compared to a batch process. Flow chemistry provides researchers the opportunity for decreased solvent utilisation through the increased implementation of solvent-free syntheses (the typical mass

transfer issues related to these conditions can be more readily overcome in flow) and more atom-efficient reactions. Considering a multistep reaction, if every single step has been optimised and performed into the same solvent the reaction could be processed in tandem in a continuous process: the reaction mixture of one step becomes the reactants for the following step creating a telescoped sequence. The telescoping of reaction pathways in flow has allowed for a decrease in waste generation by minimizing solvent switching steps and/or product isolations, handling, and manipulations of chemicals, which increase process complexity [106]. Researchers have observed a reduction of ten times in solvent consumption in their laboratories which apply flow chemistry compared to their traditional synthesis facility. The process intensification afforded by flow chemistry can enable significant improvements in yield, product quality, and efficiency [113].

Biocatalysis in flow reactors

In response to the challenges of the pharmaceutical industry and medicinal chemistry, biocatalytic flow reactors have been increasingly developed. The use of biocatalysts (both whole cells and isolated enzymes) in flow reactors allows to further increase the productivity of the process, improving at the same time the green aspect of the synthetic chemistry. Flow processing has the potential to accelerate biotransformations due to enhanced mass transfer, making large-scale production more economically feasible in significantly smaller equipment with a substantial decrease in reaction time (from hours to few minutes), and improvement in space-time yield [80]. As mentioned above, flow reactors could be of different dimensions, materials and shapes and they could be used for heterogeneous or homogeneous reactions. The whole system can be coupled to in-line monitoring systems in addition to mixing and heating devices. On-line monitoring is desirable to quickly react to fluctuations of the key parameters together with tracking the reaction's progress. Furthermore, flow reactors are easy to scale-up thanks to their modular nature. All these features combined with biocatalysts are shown in Scheme 7. Among biocatalytic flow reactors the mesoreactors and minireactors are mostly used. Two types of microfluidic chemical reactors were reported to date; the first one is chamber type and the second one is the continuous flow type. The micro chamber type has the static reagent in the micro chamber and the continuous flow type has the continuous flow of reagent through the overall microchannel [114]. The micro chamber type can be problematic when is necessary to increase the production up to gram scale. In fact, even if is a modular system, this device has a great impact on the cost of the process.



Scheme 7. Schematic representations of main components of biocatalytic flow reactors: A pumps; B reaction loops; C T-piece; D coil reactor; E column reactor; F back pressure regulator; G downstream unit; H syringe pumps; I microfluidic reactor.

Both cell-free enzymes and whole cells could be used as biocatalysts. Immobilised enzyme reactors and free enzyme reactors are the most common because cell-free systems offer advantages versus the whole cell approach, such as:

- Generally faster flow.
- Less barriers between the substrate and the catalyst.
- No need to maintain the cell wall integrityl
- Less side reactions [80].

Figure 19 shows different configurations of biocatalytic flow reactors, both for free and immobilised biocatalysts.

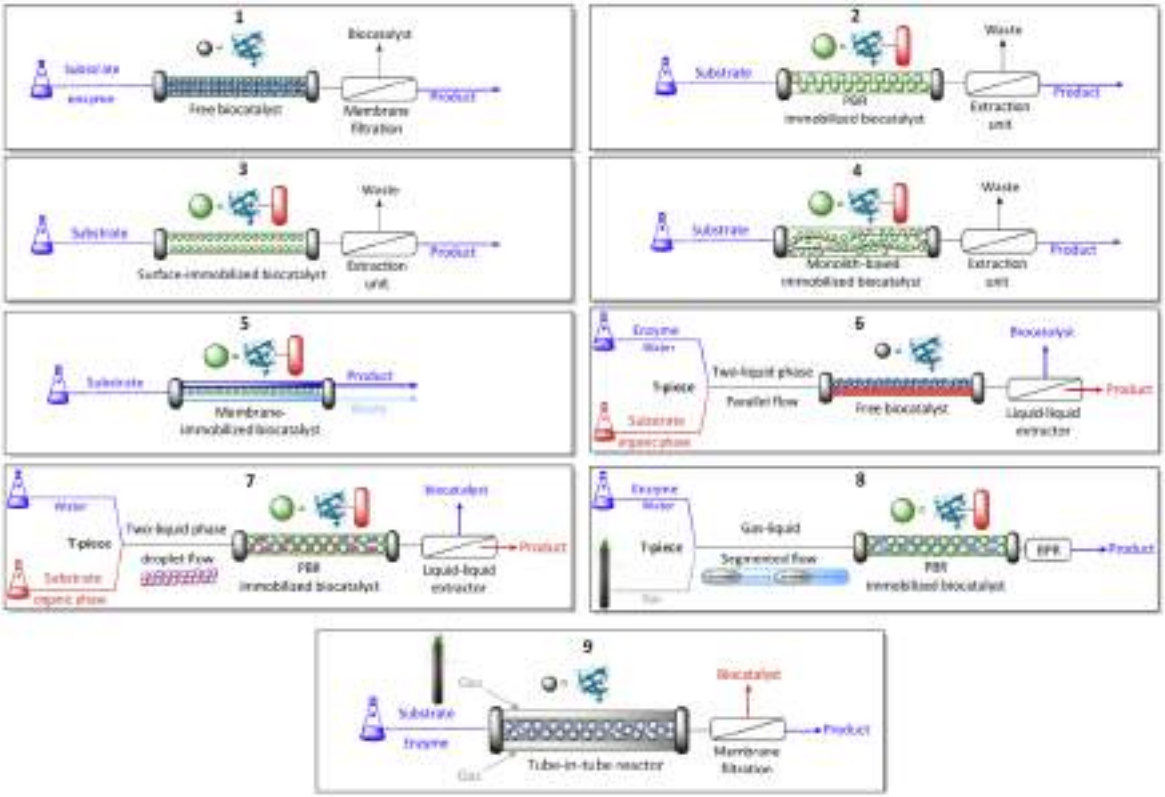


Figure 19. 1 Free biocatalyst; 2 immobilised biocatalyst in a packed bed reactor; 3 biocatalyst immobilised on the inner surface of the channel; 4 biocatalyst immobilised on a monolith; 5 biocatalyst immobilised on a membrane; 6/7 free biocatalyst/immobilised biocatalyst in a l/l biphasic parallel flow stream; 8 immobilised biocatalyst in a g/l biphasic flow stream; 9 free biocatalyst in a tube-in-tube reactor.

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Aim

In the last decade enabling technologies as continuous flow chemistry and biocatalysis became a well-established reality in academic laboratories and are slowly but constantly moving also to the industrial productions. It is not necessary anymore to demonstrate the numerous advantages related to the use of these two approaches, combined or per se, that have been already listed in the previous chapter and are universally renowned. The common misconception is that continuous chemistry and biocatalysis could be applied only to a small fraction of synthetic procedures, with numerous experimental restrictions and the necessity of expensive equipment and materials, actually slowing the spread of flow biocatalysis as a main tool in scientific research. This usually results in preventing researchers to approach new processes and relapsing to old fashioned, often unproductive and polluting, procedures.

The aim of my Doctoral Thesis was to move a step forward the demonstration of the versatility and simplicity of use of the combination of these two techniques, which are highly productive, ecological, and fully automatable (reducing the risks of human errors), in order to develop innovative tailor-made processes for the synthesis of valuable compounds. To do that, I selected different biocatalysts, in different physical forms, with which I performed the synthesis of well-defined compounds that could be useful for pharmaceutical, food and cosmetic applications, to demonstrate the broad spectrum of action of flow biocatalysis. Moreover, the products obtained were both already known compounds (*e.g.*, procaine, hydroxytyrosol) as well as new compounds that were tested for their biological properties. In all the projects, I also focused my attention on a crucial point of process optimisation: the development of in-line purification steps. The aim was to reduce downstream processes by including continuous purification steps, *i.e.*, extraction, catch and release strategies, and/or suitable immobilised scavengers.

The three main reactions that have been explored were catalysed by different classes of enzymes: reactions of functional groups transfer (from one molecule to another), redox reactions and condensation reactions for the synthesis of different functional groups (*i.e.*, esters, carbonate, carbamate). These reactions were selected due to their wide use in classical organic synthesis that usually required harsh conditions, expensive or toxic reactants, to obtain at the end the desired product with low selectivity and high environmental impact. In some cases, a combination of more than one biocatalyst was used in the same protocol to perform further modifications of the high

value chemicals obtained. Where possible the stability or the possibility to reuse the biocatalyst was evaluated in order to reduce the costs of the procedure. The choice of the right solvent was a key point in all the protocols developed and the replacement of classical organic solvent was the path followed whenever the experimental condition allowed it. This also allowed me to evaluate the operational conditions of well-known enzymes in unconventional reactions media (*e.g.*, lipase in tert-amyl alcohol or cyclopenthylmethyl ether) expanding their possible synthetic applications.

Firstly, acyl transferase from *Mycobacterium smegmatis* was employed for a greener synthesis of three different active pharmaceutical ingredients (APIs) (*i.e.*, procainamide, procaine and butacaine). This enzyme was employed in its immobilised form which allowed to stabilise and recycle the enzyme several times before a loss of activity was observed. Furthermore, the biocatalyst was able to work in pure organic solvents which was beneficial for the solubilisation of the substrates, to avoid hydrolytic side reactions and for the purification steps. In-line purifications procedure were designed and reduced drastically the manual downstream processes. The biocatalytic approach was combine with a subsequent chemical step, *i.e.*, a continuous hydrogenation. For this reaction, I took advantage by the use of a safer and continuous device (H-cube from Thales Nano) that enabled to obtain the pure final product thanks to the generation of H_2 in-situ. The aim of this work was to demonstrate the possibility to redesign well-established procedure for the synthesis of commercial APIs using continuous chemo-enzymatic approaches which permitted to reduce the ecological impact of chemical procedures, to use safer experimental conditions and to improve the automation of the whole process.

The importance of oxidation and reduction reactions in synthetic procedures is well known. Usually, chemical redox systems require long steps and harsh conditions and are still poorly selective and sustainable. On the other hand, biocatalysts involved in redox reactions are often highly selective and need mild experimental conditions. For these reasons, the second part of my doctoral project was mainly focused on the development of different redox systems mediated both by isolated enzymes commercially available (Tyrosinase from *Agaricus bisporus* and Laccase from *Trametes versicolor*) and immobilised whole cells (from *Rhodotorula rubra*). One crucial factor in the execution of redox processes in continuous reactors is the supply of oxygen since the reactions take place in a closed and monitored space. This technological problem was deeply investigated and. during my visiting period at the University of Ljubljana, I focused my attention on the development of a suitable microreactor which allowed an optimal transfer of oxygen to the biocatalyst. These projects aimed to demonstrate the feasibility of multiphasic reactions in

continuous reactors (commercially available or purposely designed) providing an enhanced control on limiting experimental parameters as oxygen supply, mass transfer, interfaces phenomena and mixing efficiency.

Lastly, I decided to explore the synthetic possibilities provided by a milestone of commercial enzymes: the lipase from *Candida antarctica* B. The literature is full of examples where the potential of this biocatalyst is exploited, in aqueous media as a hydrolytic tool and in organic solvent as a condensation catalyst. Lipase is a potent synthetic tool, has a wide action on different substrates, is thermostable and tolerant to organic solvents. Considering its application in the synthesis of esters, carbonate, and carbamate, I wondered if the ability of lipase could not be employed in greener unconventional media for the obtainment of molecules with higher lipophilicity maintaining at the same time their biological activities. The final goal was to demonstrate that biocatalysis is not confined to process optimisation, but it remains a relevant tool for explorative chemistry. Moreover, media engineering applied to biocatalysis contribute to broader the synthetic scope while further lowering the ecological impact of the process. Moreover, it is noteworthy the fact that products derived from bioprocesses starting with natural substrates are in principle defined as 'natural' if they have already been identified in plants or other natural sources (as stated by J. Schrader and colleagues[1]), providing a non-neglectable advantage in terms of food and pharma applications compared to classical chemical approaches for the synthesis of biologically active compounds.

[1] Biotechnology Letters 26: 463–472, 2004

CHAPTER 1

Transferase mediated reaction

Aciltrasferase from *Mycobacterium smegmatis* for the efficient chemo-enzymatic flow synthesis of high value amides and esters

Article on this topic:

"Efficient chemo-enzymatic flow synthesis of high value amides and esters"

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Introduction

Continuous flow synthesis of fine chemicals, such as drug substances and active pharmaceutical ingredients (APIs), is expanding rapidly in industrial contexts [1–3]. At the same time, biocatalysis is now considered one of the most promising technologies for sustainable processing in chemistry [4–6], and the combination of biocatalysis and flow chemistry has emerged as an important opportunity to expand the chemical toolbox for synthetic chemistry under environmentally benign conditions [7–10]. In this context, the development of chemo-enzymatic flow-based routes represents an innovative strategy for the obtainment of APIs by combining the advantages of the flexibility of chemical reactions with the high efficiency, regio-, chemo- and stereo-selectivity of enzymatic biotransformations [11–15]. Immobilised acyltransferase from *Mycobacterium smegmatis* (MsAcT) was recently employed for efficiently catalysing ester and amide formation in aqueous phases under flow conditions starting from primary alcohols or amines and short-chain esters [16-17]. In the present work, the catalytic power of MsAcT was also demonstrated using vinyl 4-nitrobenzoate as acyl donor in pure organic solvent. This feature appears highly beneficial to guarantee a good solubility for lipophilic substrates to achieve more productive protocols. In particular, we focused on the biocatalysed flow synthesis of amide or ester intermediates useful for the synthesis of three APIs, *i.e.*, the antiarrhythmic procainamide (**1**), and the local anaesthetics procaine (**2**) and butacaine (**3**), that were isolated in good yields after flow hydrogenation reaction (Figure 20). Common strategies for the obtainment of these APIs typically require anhydrous reaction environment, stoichiometric coupling reagents as well as harsh reaction conditions, generating a significant amount of waste [18–22]. In this context, new approaches aiming at increasing the atom economy as much as possible could be particularly interesting.

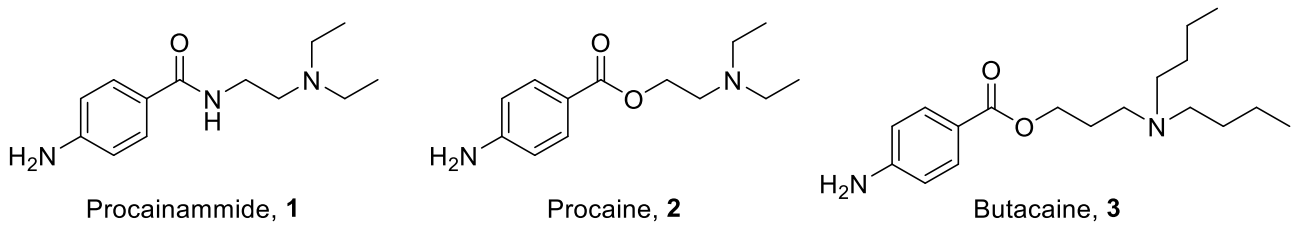


Figure 20. Chemical structure of procainamide, procaine and butacaine.

The biocatalyst

For this project, the selected biocatalyst was the acyltransferase from *Mycobacterium smegmatis* (McAcT). Is a common strategy for condensation reaction mediated by enzyme the use of lipases, which belong to the α/β hydrolase family of enzymes. Lipases are now widely used for catalytic and stereospecific transesterification reactions, in both academic and industrial laboratories [23]. The issue connected with lipases is the necessity to employ them in anhydrous environment since the presence of water will promote the hydrolytic reactions rather than the condensation. The anhydrous environment is an additional cost that must be considered in the development of a process. For this reason, the MsAcT gained attention due to its ability to favouring alcoholises over hydrolysis in water. This ability relates to the structure of the biocatalyst itself: the enzyme is an octamer where the dimers are made of two identical monomers. Each monomer is made by five β sheets and seven α helixes. The oligomeric nature of MsAcT restricts access to the active site pocket and redefines its overall topology. The intricate oligomerisation combined with the hydrophobic nature of the cannel would disfavour the entrance of water, allowing alcohol and other moieties to react with the acyl enzyme intermediate (Figure 21) [23].

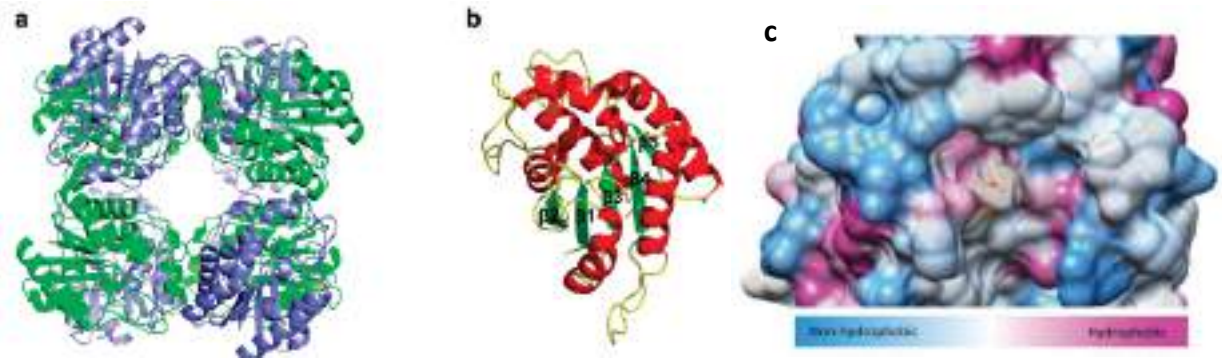
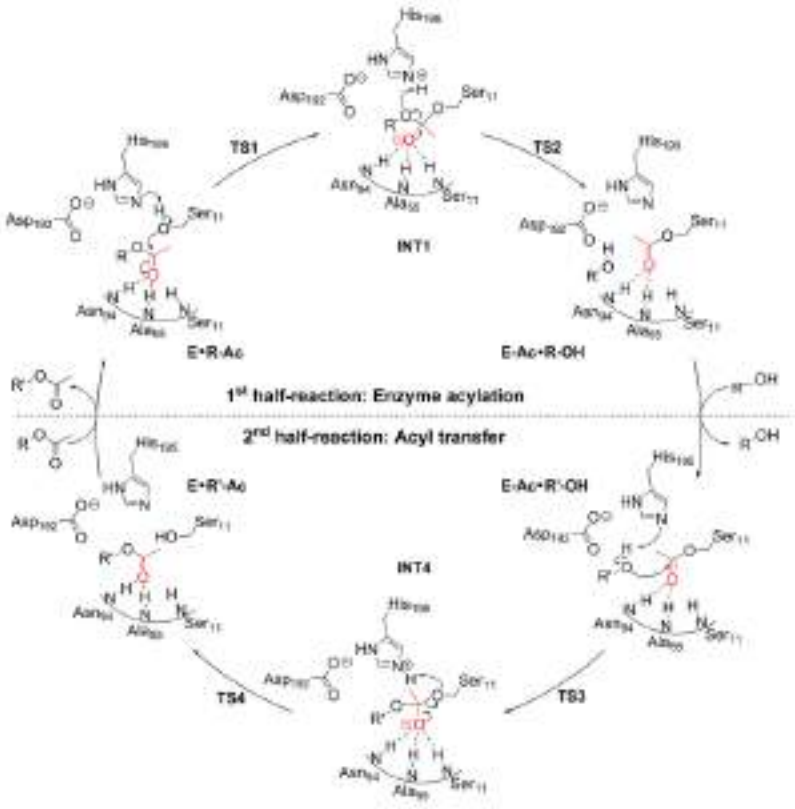


Figure 21. MsAcT structure. **a**. octamer (blue and green dimer pair) **b**. monomer with the beta sheets labelled **c**. hydrophobic tunnel which drives to the catalytic site of MsAcT; aminoacids are coloured based on their hydrophobicity [23-24]

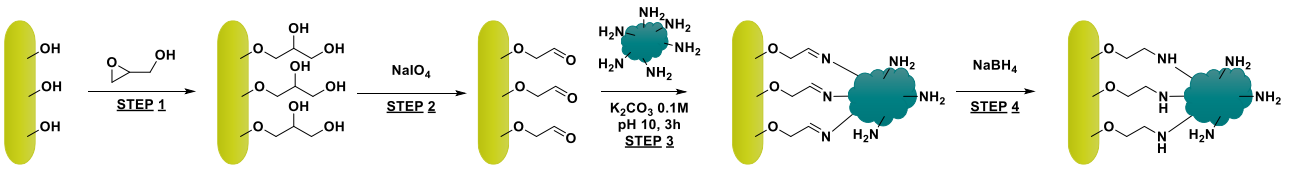
MsAcT shares similarities with the SGNH superfamily of hydrolases (*e.g.*, the octameric structure), including the catalytic triad composed of Ser11, Asp192, and His195. The mechanism of action of the acyltransferase involves four steps: firstly, the attack of the hydroxyl group of the serine takes place on the carbonyl carbon of the acyl donor substrate resulting in the acylation of the enzyme itself. Then the nucleophile enters in the enzymatic pocket and attacks the carbonyl forming the corresponding ester or amide (Scheme 8). However, even if the alcoholises reaction resulted promoted, hydrolysis remains a main side reaction that has to be considered [25].



Scheme 8. MsAcT mechanism of transesterification. [25]

MsAcT Immobilisation

To increase the enzyme stability, MsAcT was immobilised onto glyoxyl-agarose support, chosen taking into consideration a previously performed in-depth study [17].

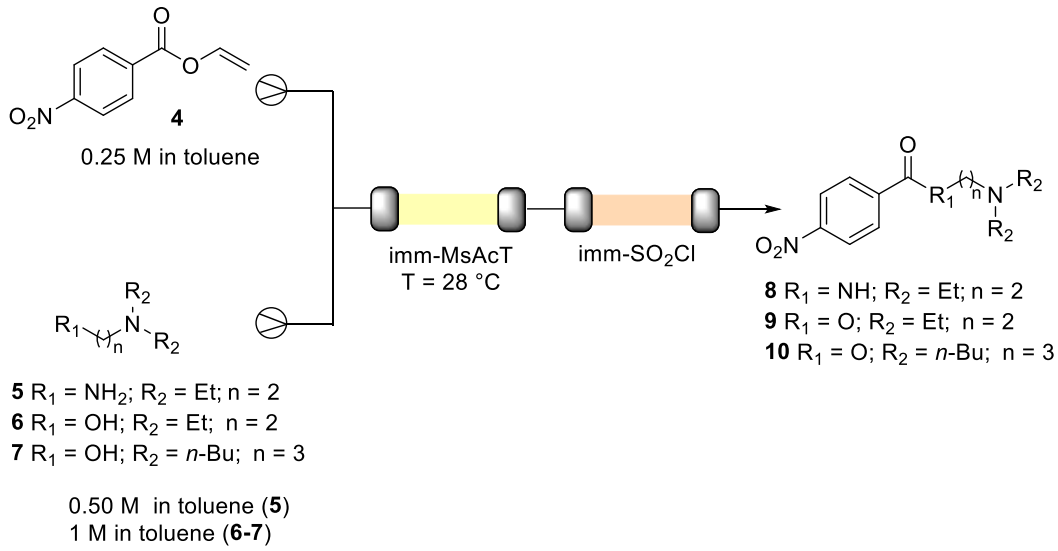


Scheme 9. Procedure for the immobilisation of MsAcT on glyoxyl-agarose.

This type of immobilisation is characterised by covalent bonds between the carrier and the enzyme. To this aim, agarose has to be properly activated through the etherification of the hydroxyl groups exposed by the support with glycidol. This step is followed by an oxidation reaction (using sodium periodate) aimed at the production of the reactive aldehydic groups. This procedure has to be performed at low temperature (4 °C) and takes approximately 20 h overall. The aldehydic groups are available to react with the free amino-groups of the enzymes: the imino bonds thus obtained are then reduced to amino bonds using sodium borohydride, in order to form a stable covalent bond between the enzyme and the support.

Results and discussion

Between different hydrophilic carriers employed (*i.e.*, agarose, cellulose, 3-aminopropylic silica, and epoxy resins), the highest recovered activity (*i.e.*, 73%) was obtained at low enzyme concentration (1 mg g_{matrix}^{-1}) using activated glyoxyl-agarose as support. Hydrophobic supports have been avoided as they could create non-specific interactions between the enzyme and non-functionalised hydrophobic regions of the carrier. Glyoxyl-agarose allowed the obtainment of a robust catalyst, easy to incorporate to flow chemistry reactors (packed bed reactor, PBR), guaranteeing regular packing and controlled fluid dynamics with consequent more-than-acceptable residence time distribution and process efficiency. In a first set of experiments, ethyl 4-nitrobenzoate was investigated as acyl donor for *N*- and *O*-acylation, but no trace of reaction was observed. Since vinyl esters are more efficient acyl donors [16-17], activated vinyl 4-nitrobenzoate **4** was prepared exploiting a Pd(II)-catalysed transvinylation reaction starting from 4-nitrobenzoic acid [26]. First, the solubility of vinyl 4-nitrobenzoate **4** was evaluated in phosphate buffer (100 mM, pH = 8) in the presence of DMSO as a co-solvent (10% v/v) (Scheme 10) [16]. However, compound **4** resulted only poorly soluble in the mixture, even increasing DMSO up to 20% v/v. Therefore, the reaction was set up in a biphasic system. In particular, the acyl donor **4** was dissolved in toluene (0.25 M) and the nucleophile, *i.e.*, N^1, N^1-diethylethane-1,2-diamine **5**, was dissolved in phosphate buffer (100 mM, pH = 8). The two phases were mixed in a T-piece to form a liquid/liquid heterogeneous segmented flow stream before entering the bioreactor kept at 28 °C. After 30 min of residence time, very low product formation was observed by HPLC (about 10%) that did not considerably increase when applying longer residence times, *i.e.*, 60 and 120 min. Moreover, a consistent amount of benzoic acid was formed (about 20% by HPLC). To limit the hydrolysis of vinyl 4-nitrobenzoate **4** and to facilitate the access of the nucleophile, we performed the biotransformation in pure organic solvent, *i.e.*, toluene [27]. Therefore, both the solutions of compound **4** and compound **5** (0.25 M and 0.50 M, respectively) were prepared in toluene, mixed in a T-piece and directed into the bioreactor (Scheme 10). Despite its bad reputation, toluene is usually reported in solvents' selection guides as a suitable option compared to other organic solvents. Moreover, the solubility of compound **4** was tested in different solvents usually considered more green (*i.e.*, 2-Me THF and CPME) but with no positive outcomes.



Scheme 10. Reaction conditions: 0.25 M solution of **4** in toluene, 0.5 M solution of the nucleophile **5**, 1 M of nucleophiles **6** and **7** in toluene; temperature of the bioreactor: T = 28 C; flow stream: toluene.

Using a 2 mL bioreactor, different residence times were evaluated (*i.e.*, 5 min, 7 min and 15 min, Table 18) and the reaction outcome was monitored by HPLC. Thanks to the high local concentration of the immobilised biocatalyst in the PBR and of the efficient mixing, using 0.25 M solution of vinyl 4-nitrobenzoate **4** and 2 equivalents of the nucleophile **5**, the reaction reached completion in only 7 min of residence time and no formation of benzoic acid was detectable by HPLC. Notably, in batch, full conversion was achieved after 24 h. Temperature was kept at 28 °C, because no beneficial effects on the conversion have been observed by increasing it. Under these conditions, the specific reaction rate reached the maximum, *i.e.*, 28 μmol min^{-1} mg_{enzyme}^{-1} resulting in being more than twice compared to the batch reaction (12 μmol min^{-1} mg_{enzyme}^{-1}).

Entry[a]	Residence Time (min)	Conversion [b]	*r* (μmol min^{-1} mg_{enzyme}^{-1}) [c]
1	5	44	18
2	7	100	28
3	15	100	13

Table 18. Effect of the residence time on the flow synthesis of *N*-(2-(diethylamino)ethyl)-4-nitrobenzamide (**8**) catalysed by immobilised MsAcT. [a] Experimental conditions: flow stream: toluene, 0.25 M solution of **4** in toluene, 0.5 M solution of **5**; reactor volume: 2 mL; immobilised MsAcT: 2.5 g with enzyme loading 1 mg/g_{enzyme}; T=28 °C; [b] Determined by HPLC. Conversions (%) are the average of two experiments; [c] Specific reactions rates (μmol min^{-1} mg_{enzyme}^{-1}) were calculated using the equations; $r = [P]*f/m_e$, where [P] is the concentration of the product (μmol mL^{-1}), f is the flow rate (mL min^{-1}) and m_e, is the amount of enzyme immobilised on the support (mg) [28], [29]

Consequently, to evaluate the stability of the bioreactor, the system was left working for 6 h under the best conditions (7 min, 28 °C, 0.25 M solution of **4** in toluene, 0.5 M solution of **5** in toluene, reactor volume 2 mL, imm-MsAcT 2.5 g with enzyme loading 1 mg/g_{enzyme}), and colleting about

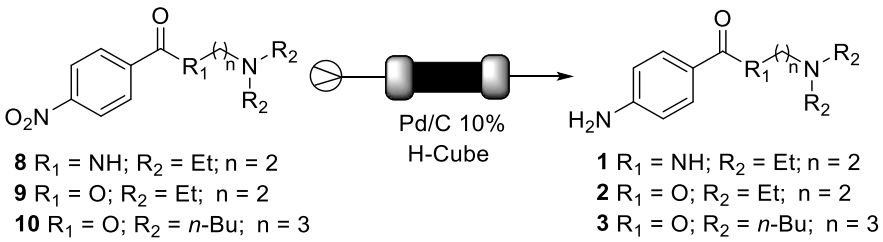
200 mL of solution, monitoring the reaction outcome every 1 hour by HPLC. Remarkably, the immobilisation strategy allowed an excellent enzymatic stability in pure toluene (average conversion: 97%); only at the end of the experiment, a slight decrease in the conversion was observed (conversion: 95%). The high performance of the immobilised enzyme in the organic environment might be ascribed to water adsorbed to the glyoxyl-agarose carrier [23]. A previous study reporting the use of the immobilised MsAcT in pure EtOAc was carried out using neopentylglycol as the substrate [30], but the use of EtOAc as solvent and acyl donor at the same time led to the inactivation of the enzyme after 4 h of continuous work due to the hydrolysis side-reaction with the formation of acetic acid and the consequent pH drop. Here, the use of toluene as main solvent to dissolve the acyl acceptors and donors reduced the hydrolysis side-reaction of the vinyl ester, thus increasing the overall yields and preserving MsAcT from inactivation due to the low pH.

To obtain the planned APIs and prove the versatility of the systems, two other nucleophiles, *i.e.*, 2-(diethylamino)ethanol **6** and 3-(dibutylamino)propan-1-ol **7**, have been exploited under the previously used conditions (residence time:7 min; T = 28 °C), using vinyl-4-nitrobenzoate **4** as acyl donor (1 eq.). Being the primary alcohols less nucleophilic than the primary amine previously used, a very low conversion was achieved in 7 min of residence time, using two equivalents of compound **6** or **7**. Then, to achieve higher conversions, the residence time was increased, but even after 60 min only 32% of conversion was obtained. Therefore, the stoichiometry and the residence time were modified (Table 19). The concentration of the nucleophile was increased up to 1 M and different residence times have been tested (*i.e.*, 7, 15 and 30 min, Table 19). The best conversion was achieved by using four equivalents of the nucleophile in 15 min of residence time (Table 19, entries 4 and 6). A further increase in the residence time was not beneficial (Table 19, entries 5 and 7).

Entry [a]	Nucleophile	Nucleophile concentration (M)	Residence time (min)	Conversion [b] (%)
1	6	0.5	7	25
2	7	0.5	7	15
3	6	1	7	32
4	6	1	15	72
5	6	1	30	74
6	7	1	15	36
7	7	1	30	37

Table 19. Synthesis of esters **9** and **10** catalysed by immobilised MsAcT. [a] Experimental conditions: flow stream: toluene; 0.25 M solution of **4** in toluene, reactor volume: 2 mL; immobilised MsAcT: 2.5 g with an enzyme loading of 1 mg/$g_{agarose}$; T = 28 °C; [b] Determined by HPLC. Conversions (%) are the average of two experiments.

The process was further implemented with an in-line purification procedure using immobilised sulphonyl chloride that was packed into a reactor column connected with the bioreactor (Scheme 10) that efficiently trapped the excess of the nucleophile. In this way, compounds **8**, **9** and **10** were isolated without any further purification, after simple solvent evaporation in moderate to good yields (yields: 93%, 68% and 32%, respectively), thus increasing the automation of the protocol and reducing the time associated with work-up procedures. The obtained intermediates **8**, **9** and **10** were then submitted to a flow hydrogenation reaction using a 10% Pd/C cartridge to reduce the nitro group to aniline. To avoid transesterification reaction of esters **9** and **10** using methanol as the solvent, ethyl acetate was used (Scheme 11). The reduction resulted complete working at 60 °C and 10 bar at 0.8 mL/min. The desired APIs, **1**, **2**, and **3**, were isolated after solvent evaporation in quantitative yields. Unluckily, the different solvents required for the hydrogenation step prevent the possibility of telescoping the acylation step with the reduction reaction. However, the steps were highly automated thanks to the use of integrated software.



Scheme 11. Synthesis of procainamide (**1**), procaine (**2**) and butacaine (**3**). Reaction conditions: solution of compound **8**, **9** and **10** in EtOAc (50 mg/mL), T = 60 °C, P = 10 bar.

Materials and methods

All reagents and solvents were purchased from commercial suppliers and were used without further purification. The continuous flow biotransformations were performed using a R2C/R4 flow reactor commercially available from Vapourtec equipped with Omnifit glass columns (10 mm i.d. x 100 mm length; 6.6 mm x 100 mm length). Hydrogenation reactions were recorded using an H-cube Mini Plus reactor (ThalesNano). ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 (300 mHz) spectrometer. Chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) are expressed in Hz. HPLC analyses were performed using Waters 1525 Binary HPLC Pump, equipped with Waters 2489 UV-vis detector (Waters, Milford MA) and Ascentis C18 column (25 cm x 4 mm, 4 µm particle size). Protein expression, protein purification and free enzyme activity measurements were performed following previously reported protocols [31].

Analytical Method

Mobile phase: H2O + 0.05% TFA(A)/ACN + 0.05% (B); gradient conditions: 0–5 min 80% (A)/20% (B), 5–8 min 20% (A)/80% (B); flow rate: 1.0 mL min^{-1}; λ: 254 nm. Injection volume: 10 µL; Reaction samples were diluted with a solution 1:50 H_2O/ACN + 0.05% TFA. Retention times (t_R): 4-nitrobenzoic acid = 5.17 min, vinyl 4-nitrobenzoate (**4**) = 6.14 min, *N*-(2-(diethylamino)ethyl)-4-nitrobenzamide (**8**) = 2.03 min, 2-(diethylamino)ethyl 4-nitrobenzoate (**9**) = 3.03 min, 3-(dibutylamino)propyl 4-nitrobenzoate (**10**) = 5.21 min.

Glyoxyl-agarose activation

5 g of Sepharose CL-6B were filtered and washed with deionised water. Once dried the Sepharose was transferred into a Falcon tube kept in ice. 1.425 mL of deionised water, 2.4 mL of NaOH 1.7 M, 68.4 mg of $NaBH_4$ and 1.7 mL of glycidol were added at 0° C. The mixture was mechanically stirred for 30 min at 0° C and then at room temperature for 18 h. Glyoxyl-agarose was then filtered, washed with deionised water and transferred into a solution of $NaIO_4$ 100 mM (34.3 mL). The mixture was stirred for 2 h, filtered, washed with deionised water and stored at 4° C [32].

MsAcT immobilisation on glyoxyl-agarose

2.5g of glyoxyl-agarose were added to a solution of MsAcT in carbonate buffer (50 mM, pH = 10, 35 mL). For every gram of support was added 1 mg of enzyme. The mixture was stirred for 24 h at room temperature. 35 mg of $NaBH_4$ were added and the mixture was stirred for 30 min. The mixture was then filtered, washed with phosphate buffer 10 mM pH 5 and deionised water [17].

Synthesis of Vinyl-4-nitrobenzoate (**4**) [26, 33]

To a solution of *p*-nitrobenzoic acid (5.0 mmol) in THF (5 mL), vinyl acetate (80 mmol, 7.5 mL) and palladium(II) acetate (0.5 mmol) were added. After degassing, the reaction mixture was stirred for 30 min at room temperature. Then, 10 % w/w of sulfuric acid in THF (5 drops) was added and the mixture was stirred at 60 °C overnight, then filtered through celite. The solvent was evaporated, and the crude mixture was purified by column chromatography (cyclohexane/ EtOAc 9:1) as a pale-yellow solid. Mp: 74–76 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 8.38–8.24 (m, 4H), 7.51 (dd, *J* = 13.9, 6.2 Hz, 1H), 5.15 (dd, *J* = 13.9, 2.0 Hz, 1H), 4.81 (dd, *J* = 6.2, 2.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): 161.9, 150.9, 141.2, 134.4, 131.2, 123.8, 99.6.

Biocatalysed flow synthesis of *N*-(2-(diethylamino)ethyl)-4-nitrobenzamide (**8**) [34]

A glass column (6.6 mm i.d.) was packed with 2.5 g of imm-MsAcT (1 mg/g). A 0.25 M solution of vinyl 4-nitrobenzoate (**4**) in toluene and a 0.5 M solution of amine **5** in toluene were prepared. The two solutions (10 mL each) were mixed in a T-piece and the resulting flow stream was directed into the bioreactor (packed bed reactor volume: 2.0 mL). The temperature was set at 28 °C, at ambient pressure, and the flow rate was set at 143 µL/min for each pump. The exiting flow stream was directed into a column (6.6 mm i.d.) packed with sulphonyl chloride polymer-bound 70–90 mesh (1.2 g, 2.5–3.0 mmol/g). The columns were then washed with 4 mL of toluene to fully recover the product. The solution was collected, and the solvent was evaporated under reduced pressure to isolate amide **8**. Yield: 93%. ^{1}H NMR (300 MHz, $CDCl_3$) 8.30–8.25 (m, 2H), 8.00–7.93 (m, 2H), 7.35 (bs, 1H), 3.58–3.48 (m, 2H), 2.73–2.65 (m, 2H), 2.59 (q, *J* = 7.2 Hz, 4H), 1.11 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) 165.1, 149.5, 139.9, 128.2, 123.7, 51.4, 47.0, 37.0, 11.3.

Biocatalysed flow synthesis of 2-(diethylamino)ethyl 4-nitrobenzoate (**9**) [35] and 3-(dibutylamino)propyl 4-nitrobenzoate (**10**)

A glass column (6.6 mm i.d.) was packed with 2.5 g of imm-MsAcT (1 mg/g). A 0.25 M solution of vinyl 4-nitrobenzoate (**4**) in toluene and a 1 M solution of alcohol **6** and **7** in toluene were prepared. The two solutions (10 mL each) were mixed in a T-piece and the resulting flow stream was directed into the bioreactor (packed bed reactor volume: 2.0 mL). The temperature was set at 28 °C, at ambient pressure and the flow rate was set at 67 µL/min for each pump. The exiting flow stream was directed into a column (10 mm i.d.) packed with sulphonyl chloride polymer-bound 70–90 mesh (3.5 g, 2.5–3.0 mmol/g). The columns were then washed with 4 mL of toluene to fully recover the product. The solution was collected and the solvent was evaporated under reduced pressure to

isolate the desired ester **9** and **10**. 2-(diethylamino)ethyl 4-nitrobenzoate (**9**) Yield: 68%. ^{1}H NMR (300 MHz, $CDCl_3$) 8.28–8.22 (m, 2H), 8.20–8.14 (m, 2H), 4.45–4.38 (m, 2H), 2.85–2.80 (m, 2H), 2.63–2.55 (m, 4H), 1.03 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) 164.6, 150.4, 135.7, 130.6, 123.4, 64.2, 50.9, 47.8, 12.0. 3-(dibutylamino)propyl 4-nitrobenzoate (**10**). Yield: 32%. ^{1}H NMR (300 MHz, $CDCl_3$) 8.32–8.25 (m, 2H), 8.23–8.18 (m, 2H), 4.48–4.40 (m, 2H), 2.62–2.55 (m, 2H), 2.50–2.38 (m, 4H), 2.00–1.87 (m, 2H), 1.45–1.35 (m, 4H), 1.35–1.22 (m, 4H), 0.89 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) 164.5, 150.5, 135.3, 130.7, 123.5, 63.8, 52.6, 49.8, 26.8, 24.7, 20.2, 13.7.

Synthesis of Procainammide (1) [36], Procaine (2) [37] and Butacaine (3)

A solution of compound **8**, **9** and **10** was prepared in EtOAc (50 mg/mL) and was submitted to hydrogenation reaction in a H-Cube Mini at 0.8 mL/min, T = 60 °C and P = 10 bar. The solvent was evaporated to obtain the desired products in quantitative yields. Procainamide (**1**): ^{1}H NMR (300 MHz, $CDCl_3$) 7.63–7.57 (m, 2H), 7.20 (bs, 1H), 6.64–6.57 (m, 2H), 4.10 (bs, 2H), 3.50–3.40 (m, 2H), 2.70–2.63 (m, 2H), 2.61 (q, *J* = 7.2 Hz, 4H), 1.04 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) 169.4, 149.8, 128.7, 123.6, 114.0, 51.6, 47.0, 36.7, 11.0. Procaine (**2**): ^{1}H NMR (300 MHz, $CDCl_3$) 7.85–7.79 (m, 2H), 6.64–6.57 (m, 2H), 4.40–4.35 (m, 2H), 4.15 (bs, 2H), 2.90–2.80 (m, 2H), 2.70–2.60 (m, 4H), 1.07 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) 166.6, 151.0, 131.6, 119.4, 113.73, 62.2, 50.8, 47.5, 11.5. Butacaine (**3**): ^{1}H NMR (300 MHz, $CDCl_3$) 7.88–7.81 (m, 2H), 6.67–6.60 (m, 2H), 4.35–4.25 (m, 2H), 4.07 (bs, 2H), 2.65–2.55 (m, 2H), 2.50–2.40 (m, 4H), 1.97–1.84 (m, 2H), 1.50–1.40 (m, 4H), 1.40–1.23 (m, 4H), 0.90 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) 166.6, 150.8, 131.5, 119.8, 113.7, 62.6, 53.7, 50.3, 28.8, 26.1, 20.6, 14.0.

Conclusions

An efficient flow-based biocatalytic protocol characterised by high rates and high substrate loading has been developed for the obtainment of pharmaceutically relevant intermediates. In line with the increasing emphasis placed today on biologically mediated chemical reactions, the system exploits the glyoxyl-agarose covalent immobilisation of MsAcT, a versatile acyltransferase from *Mycobacterium smegmatis*. Immobilised MsAcT was employed in pure organic solvent (*i.e.*, toluene), demonstrating high stability and reusability, reducing the cost contribution of the biocatalyst preparation to the final product, and making the enzyme useful for different synthetic applications performed in organic solvents. Notably, using a 2 mL bioreactor (*i.e.*, 2.5 mg of MsAcT), about 6.4 g of amide **8** were obtained with a productivity of 0.43 g h^{-1} mg_{enzyme}^{-1}, demonstrating the applicability of the system for gram scale synthesis. Flow processing dramatically accelerated biotransformations making the production of three APIs, *i.e.*, the antiarrhythmic procainamide, and the local anaesthetic drugs procaine and butacaine, easily feasible with a substantial reduction in reaction time (7–15 min) compared to batch processes (24 h). In-line purification of the key amide and ester intermediates was added downstream, enhancing the automation of the process. A final hydrogenation step using the H-Cube reactor was further carried out to obtain the desired molecules with excellent yields (>99%) making the process fast, safe and easily handled. Due to the availability of larger packed bed reactors and the robustness of the proposed immobilisation protocol, the applicability on a larger scale seems to be feasible; however, deeper calculations using enzyme activity, stability (cycles) and cost data of the supported enzyme and scavenger are necessary to demonstrate that very high-priced chemicals might be produced economically under such conditions.

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CHAPTER 2

Redox biotransformations

i) Tyrosinase from *Agaricus bisporus* for the biocatalysed oxidation of tyrosol into high value hydroxytyrosol combined with their acetate esters production mediated by immobilised MsAcT for a full automated continuous process

ii) Whole cells of *Rhodotorula rubra* for the enantioselective reduction of prochiral ketones: development of a tailor-made bioreactor for the continuous production of relevant pharmaceutical intermediates

iii) Development of a novel microreactor-based process for continuous biocatalysed synthesis of a biologically active synthetic magnolol derivative mediated by a laccase from *Trametes versicolor*

i) Tyrosinase from *Agaricus bisporus* for the biocatalysed oxidation of tyrosol into high value hydroxytyrosol combined with their acetate esters production mediated by immobilised MsAcT for a full automated continuous process

Article on this topic:

"Biocatalyzed flow oxidation of tyrosol to hydroxytyrosol and efficient production of their acetate esters"

F. Annunziata[1], M. L. Contente[2], C. Pinna[2], L. Tamborini[1], A. Pinto[2] – *Antioxidants*, **2021**, 10, 1142, DOI: 10.3390/antiox10071142

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Introduction

The demand for nontoxic antioxidants that are active in hydrophilic and lipophilic systems has led to the search for natural antioxidants that can be used in oil-based formulas and emulsions. Tyrosol (4-hydroxyphenethyl alcohol, Ty, Figure 22) and hydroxytyrosol [2-(3,4-dihydroxyphenyl)ethanol, HTy, Figure 22] are valuable dietary phenolic compounds present in olive oil and wine and are shown to possess a range of biological effects, including antioxidant, anti-inflammatory, cardioprotective, neuroprotective, anticancer, antidiabetic, and antimicrobial properties [1–4]. Particularly, HTy is one of the most powerful natural antioxidants due to the presence of the *o*-dihydroxyphenyl moiety [5]. Importantly, toxicological studies demonstrated that HTy is non-genotoxic and non-mutagenic [6], [7], raising the possibility of using this compound as a nutraceutical [8–10]. It is employed in the food (stabiliser for vegetable oils, beverages, margarines, yogurts, etc.), pharmaceutical (supplements), and cosmetic (sunscreens, lotions, shampoos, deodorizers, etc.) industries. In particular, cosmetic applications of HTy as an anti-aging and anti-inflammatory ingredient have been reported [11].

Considering the industrial applications of HTy, as well as the studies on its biological properties, it is important to have at hand sustainable and efficient synthetic procedures at competitive prices to prepare this compound. Therefore, it is not surprising that many chemical efforts have been made to collect pure HTy, either by synthesis or from natural sources [12]. Particularly, HTy can be obtained from Ty chemically, often using costly reactants [13–15]; enzymatically [16]; or from tyrosine through three biocatalysed steps[17].

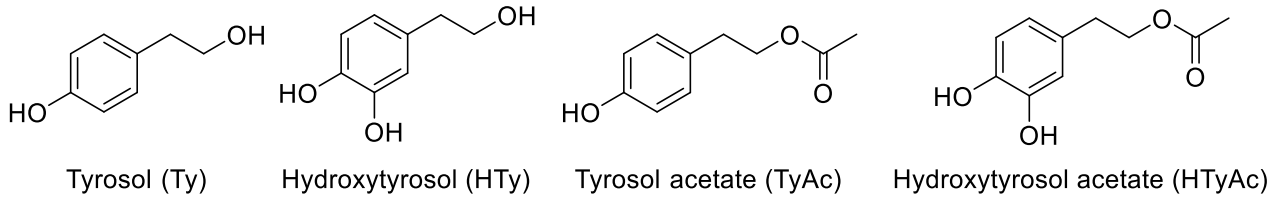


Figure 22. Chemical structure of tyrosol, hydroxytyrosol, tyrosol acetate and hydroxytyrosol acetate.

Moreover, the acetylation of the primary alcohol of Ty and HTy, to give TyAc and HTyAc (Figure 22), respectively, increases their lipophilicity and lipid solubility, modifying their bioavailability [18], stability [19] and antioxidant effect in cosmetic products and food emulsions [20]. HTyAc was described for the first time in virgin olive oils by Brenes et al.[21]. It has been reported that the acetylation of HTy significantly increases its transport across the small intestinal epithelial cell barrier, offering enhanced bioavailability compared to HTy; consequently, acetylation represents a

strategy that can be applied to improve the absorption of polyphenols, thus increasing their potential biological activity [18], [22–24]. In fact, several studies have reported the antioxidative, anti-inflammatory, neuroprotective and antiarthritic effects of HTyAc [25–28]. The acetylation reaction can be performed chemically, with acid chlorides or acid anhydrides, but these routes do not meet the requirements necessary for food applications. The use of enzymes in non-aqueous media can overcome this issue. Over the years, the enzymatic esterification of phenolic alcohols has been reported, mainly using lipases [29-30].

In this context, the possibility to have access to pure HTy and its corresponding acetate derivative HTyAc (Figure 1) is very appealing. Therefore, the aim of the present work is the design of sustainable protocols for the biocatalysed synthesis of high-value HTy starting from easily accessible Ty, and for the obtainment of their acetate derivatives, characterised by increased lipophilicity.Two biocatalysts were selected: a commercially available free tyrosinase from *Agaricus bisporus* for the regioselective oxidation of Ty to HTy; and an immobilised acyltransferase from *Mycobacterium smegmatis* (MsAcT) [31-32], for the efficient and selective acetylation of the primary alcohol of Ty and HTy.

The biocatalyst

Tyrosinase is a copper-containing enzyme which is widely distributed in nature, from microorganism and animals to plants. Tyrosinase (EC 1.14.18.1) is a polyphenol-oxidase that catalyses the hydroxylation of monophenols to *o*-diphenols and the oxidation of catechols to *o*-quinones. The addition of ascorbic acid (or other reducing agents) results in the increased accumulation of catechol, reducing quinones formed by tyrosinase activity back to catechol [33]. In particular, tyrosinases derived from mushrooms has gained attention and became popular since they are readily available and useful in a number of applications. For this reason, for this project, a commercial tyrosinase from from *Agaricus bisporus* was selected to oxidase Ty into HTy. Tyrosinase from *A. bisporus* was reported to be a heterotetramer comprising two heavy (H) and light (L) chains with a molecular mass of 120 kDa. The complete sequence has been established: the enzyme contains three domains of which the central domain contains two Cu binding sites, called Cu_A and Cu_B. Six conserved histidine residues bind a pair of copper ions in the active site of the enzyme tyrosinase, which interact with both molecular oxygen and its phenolic substrate (Figure 23). The location of cysteine (Cys) also plays an important role in the formation of disulfide linkages, which stabilise protein structure.

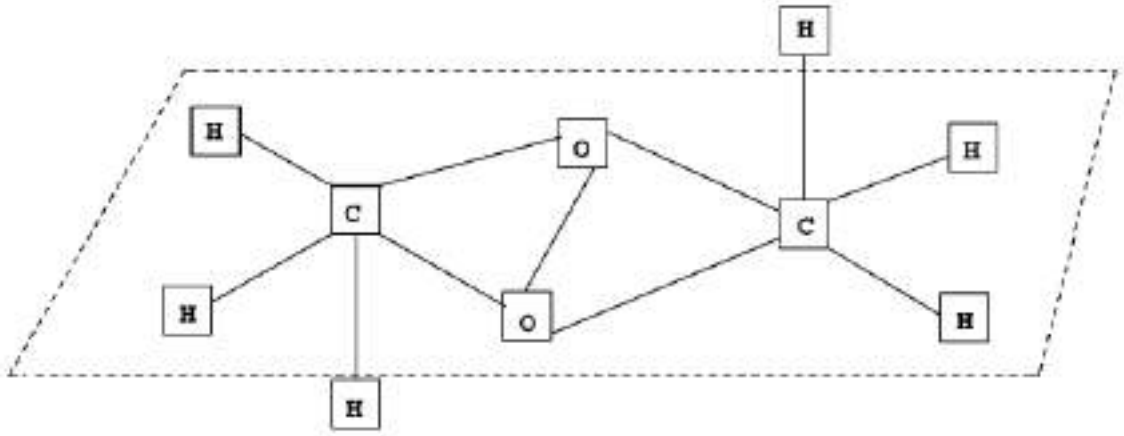
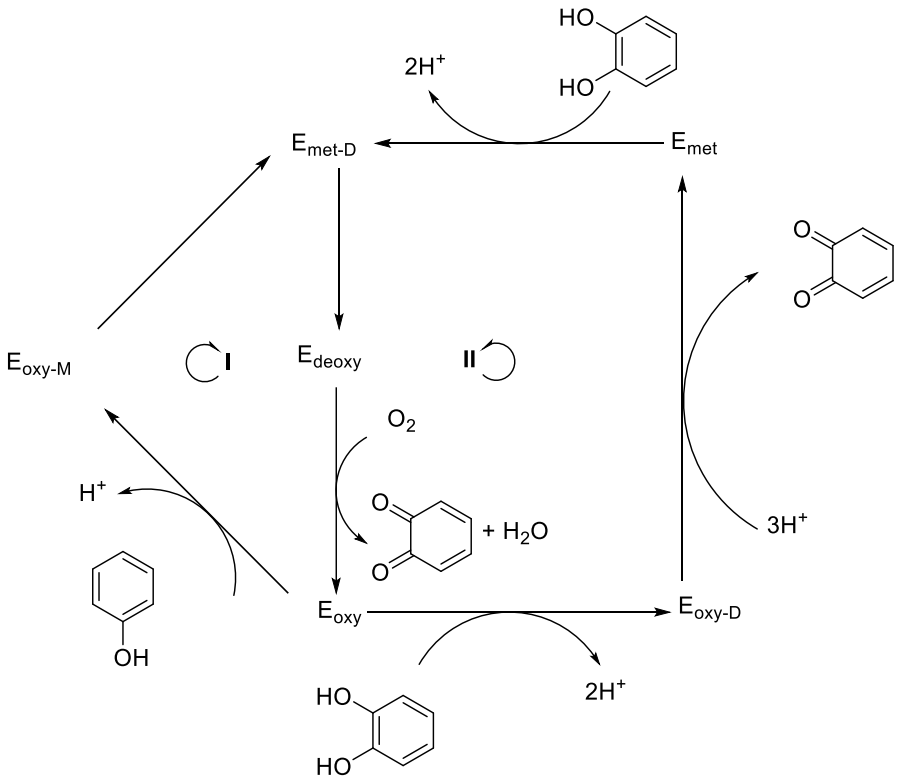


Figure 23. Schematic representation of binuclear copper site. C) Cu ion, O) oxygen, and H) His-N

As previously mentioned, tyrosinase catalyses two different oxidations (Scheme 12). In the first cycle, the oxidation of monophenols to cathecols is performed by oxygen and the enzyme passes through four different states E_{deoxy}, E_{oxy}, $E_{oxy\text{-}M}$, and $E_{met\text{-}D}$. In the second cycle, the cathecols are further oxidised as the enzyme passes through five enzyme states (E_{deoxy}, E_{oxy}, $E_{oxy\text{-}D}$, E_{met}, and $E_{met\text{-}D}$). The two cycles lead to the formation of *o*-quinones, which spontaneously react with each other to form oligomers [34].



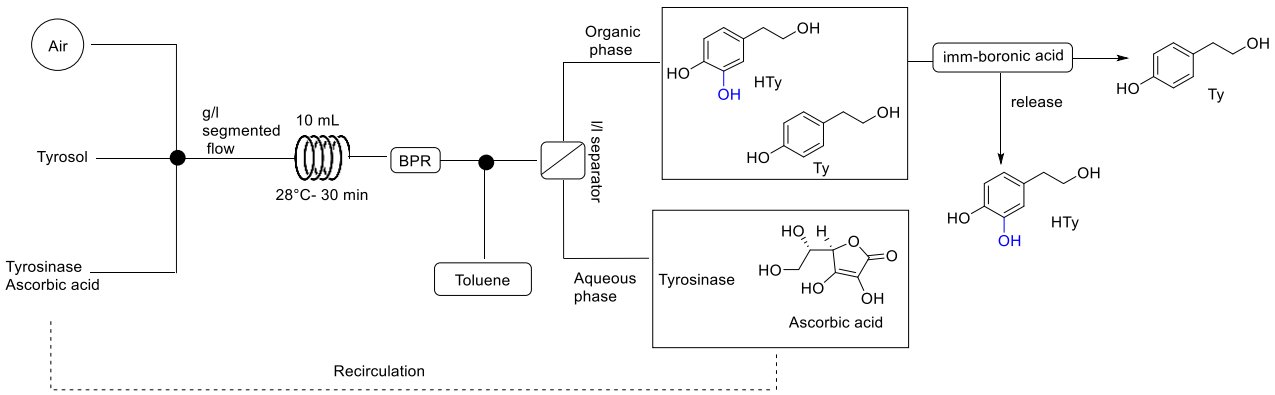
Scheme 12. Catalytic cycles of tyrosinase.

Results and discussion

The first relevant thing to understand was the role of O_2: in particular, if the air present in the flow stream was enough for the enzyme to perform the oxidation or was necessary to add a gas inlet. The conversion obtained flowing the stock solutions (tyrosol 20 mM and ascorbic acid 40 mM) without a specific inlet of air brought to a very low conversion (25 % in 60 min). Increasing the amount of biocatalyst was not beneficial for the overall conversion. Since it was evident that the conversion into desired product HTy was directly linked to the amount of O_2 in the reaction environment, to guarantee the oxygen supply two technical solutions have been evaluated: the use of a tube-in-tube reactor, specifically designed for the execution of reactions involving the presence of a gas reagent [35], and a segmented air/liquid flow stream in a tubular PTFE reactor. In this last case, flow-based reactors may offer advantages when performing multiphase reactions, including gas/liquid or liquid/liquid reactions, due to facilitated mass transfer and increased interfacial area for the exchange of chemical species [36–39]. The first experiments were performed using reaction conditions (*i.e.*, substrate concentration, stoichiometry, solvent and temperature) reported for the batch reaction [40]. The conversion obtained by flowing the solutions of the starting materials without a specific inlet of air brought a very low conversion (*i.e.*, 25% molar conversion in 60 min of residence time, Table 20, Entry 1), even when saturating the stock solutions. Using a tube-in-tube reactor, a 40% conversion was achieved (Table 20, Entry 2). The best configuration was obtained by adding an inlet of air, forming an air/liquid biphasic system (Scheme 13); in this way, the conversion increased to 47% (Table 20, Entry 3). An increase in the stoichiometric ratio in favour of ascorbic acid (from 1:2 to 1:5) avoided the formation of overoxidised by-products, resulting in a cleaner reaction with similar conversion (*i.e.*, 48%, Table 20, Entry 4). A reduction in the substrate concentration (from 20 mM to 10 mM) allowed the achievement of 78% conversion in 30 min of residence time (Table 20, Entry 6), which was 2.9 fold the one obtained in batch in 24 h. The higher reaction performance obtained in flow conditions can be explained considering the more efficient mixing and mass transfer in the biphasic system, the better control of reaction parameters (*e.g.*, residence time, temperature) and the circumvention of some critical issues of batch biotransformations such as substrate/product inhibition effects.

Entry	Ty(mM)	Ascorbic acid (mM)	Residence time (min)	Tyrosinase (U/mL)	m. c. (%)
1[a]	20	40	60	300	25
2[b]	20	40	60	300	40
3[c]	20	40	60	300	47
4[c]	20	100	60	300	48
5[c]	10	50	60	300	76
6[c]	10	50	30	300	78
7[c]	10	50	15	300	50

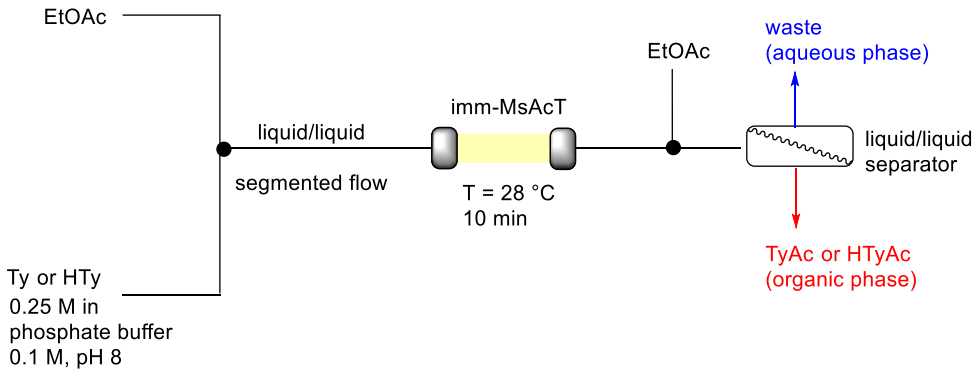
Table 20. Optimisation of reaction parameters. The solutions of Ty and ascorbic acid have been prepared in sodium phosphate buffer 0.1 M pH 7.0. The reactions have been performed at 28 °C. Conversions have been determined by ^{1}H NMR after extraction with toluene and evaporation of the solvent. [a] Reactor: 10 mL PTFE coil; [b] Reactor: 15 mL tube-in-tube reactor; [c] Reactor: 10 mL PTFE coil; air/liquid segmented flow; m. c. = molar conversion.



Scheme 13. Optimised flow reactor configuration for the bio-oxidation of Ty to HTy and in-line purification. The solutions of Ty and tyrosinase with ascorbic acid were prepared in sodium phosphate buffer 0.1 M, pH 7.0. BPR: 40 psi

The aqueous flow stream was then extracted in-line by adding an inlet of toluene. The two phases were separated using a liquid/liquid separator to recirculate the wastewater containing the biocatalyst and the excess ascorbic acid, thus creating a self-sufficient closed-loop system with improved efficiency. The aqueous phase was recirculated three times with only a slight reduction in the conversion (cycle 1: 78% m.c.; cycle 2: 74% m.c.; cycle 3: 69% m.c.). After the third cycle, a significant reduction in the conversion (56%) was observed, even when adding fresh ascorbic acid to the reservoir solution. Moreover, the organic phase containing HTy and unreacted Ty was purified in-line thanks to a catch-and-release procedure. This strategy, involving supported boronic acids able to trap HTy through the formation of a cyclic borate with the catechol group, leaves the unreacted Ty in the exiting flow stream. The HTy was then released using an acidic solution (2 N

HCl). After the optimisation of continuous production of HTy, the synthesis of TyAc and HTyAc was performed. To obtain the corresponding acetate derivatives, a bioreactor packed with an immobilised acyltransferase from *Mycobacterium smegmatis* (MsAcT) was used (Scheme 14). As previously described, MsAcT shows a characteristic hydrophobic tunnel leading to the active site that can disfavour the ingress of water, thus promoting ester formation over hydrolysis also in aqueous media. The system exploited the covalent immobilisation of MsAcT onto agarose beads, increasing the robustness and longevity of the immobilised biocatalyst (enzyme loading 1 mg g_{matrix}^{-1}). The inlet system was composed with an aqueous solution of Ty or HTy (0.25 M) and an organic phase (pure EtOAc as acetyl donor); the two phases were mixed in a T-piece to form a liquid/liquid heterogeneous segmented flow stream before entering the column. Reactions were performed at 28 °C in phosphate buffer (0.1 M, pH 8.0). Connected software was used to realise an automated process for the collection of the product at the steady state.



Scheme 14. Reactor configuration for the flow-based acylation of Ty and HTy. Column reactor i.d. = 6.6. mm; bioreactor volume = 2.0 mL.

Notably, primary alcohol acetylation occurred with complete chemo selectivity, since no reaction involving the phenolic OH was observed, demonstrating that our process is a clean, mild and virtually zero-waste procedure. Full conversion of Ty was achieved after 10 min of residence time using a 7:3 ratio of substrate solution/acetyl donor (EtOAc). A noteworthy observation was that the batch biotransformation did not go to completion in 24 h. An inlet of EtOAc and an in-line liquid/liquid separator were introduced downstream of the process (Scheme 14) for the collection of the organic/aqueous phase. The pure TyAc was obtained by flash chromatography (81% isolated yield). Under the same conditions, HTyAc was isolated in 75% yield. Immobilisation coupled with continuous removal of the products gave high stability to the biocatalyst under operating conditions. Moreover, the use of a flow environment associated with a two-liquid phase system avoids the formation of emulsions difficult to resolve, often present under conventional stirring.

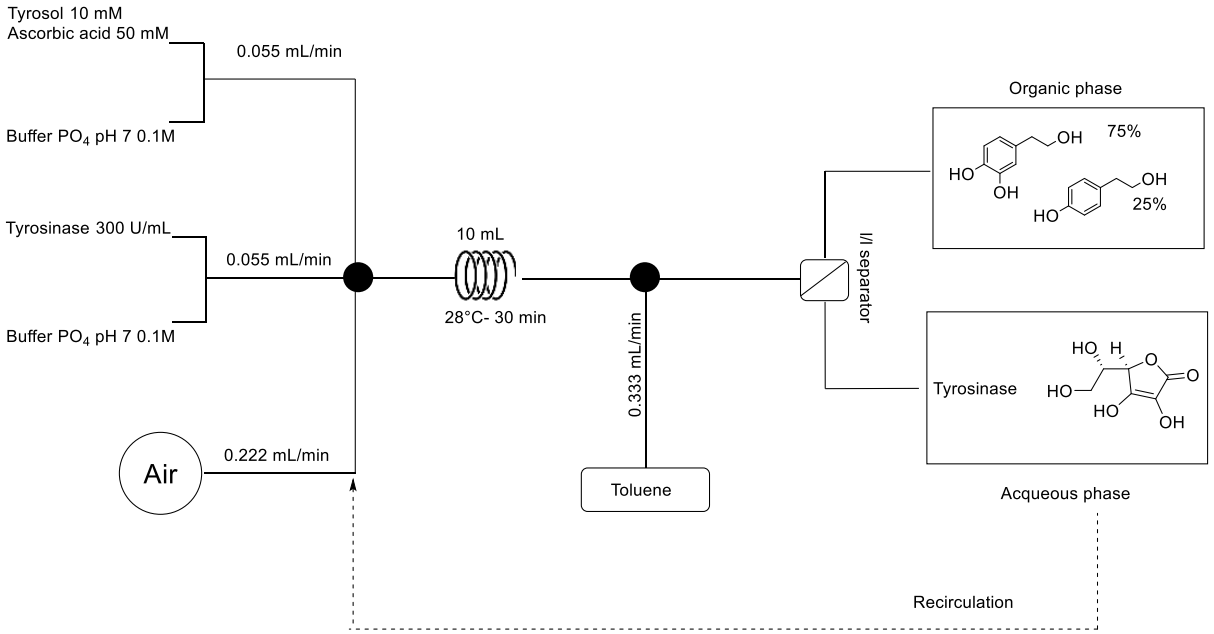
Materials and methods

Reagents and solvents were obtained from commercial suppliers and were used without further purification. NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer using the residual signal of the deuterated solvent as the internal standard. ^{1}H chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in hertz (Hz). Continuous flow biotransformations were performed using an R2+/R4 or a Series E Vapourtec flow reactor equipped with an Omnifit® (Merck, Milan, Italy) glass column (6.6 mm i.d. x 100 mm length), a PTFE coil (10 mL) or a tube-in-tube reactor (15 mL). The temperature sensor sat on the wall of the reactors. Pressure was controlled by using backpressure regulators. For the in-line extraction, an additional HPLC pump (ThalesNano, Stepbio, Bologna, Italy) was used. In-line liquid/liquid separations were performed using a Zaiput separator. TLC analyses were performed on commercial silica gel 60 F254 aluminium sheets; spots were further evidenced by spraying with a dilute alkaline solution of $KMnO_4$. Mushroom tyrosinase from *Agaricus bisporus* (8503 U/mg), sodium phosphate monobasic, sodium phosphate dibasic, ethyl acetate, toluene, sodium sulfate anhydrous, polymer-supported boronic acid and molecular sieves were purchased from Merck (Milan, Italy); tyrosol and ascorbic acid were purchased from Fluorochem (Zentek, Milan, Italy). For MsAcT, protein expression and purification, and free enzyme activity measurements (150 U/mg) were performed following previously reported protocols [41]. One unit (U) of activity is defined as the amount of enzyme which catalysis the consumption of 1 mol of substrate per minute. Aldehyde agarose immobilisation and immobilised MsAcT activity measurements (120 U/mg) were performed as described in chapter 1 [32].

Batch oxidation of tyrosol by free tyrosinase from *Agaricus bisporus*

The oxidation of Ty into HTy in batch mode was performed according to the method reported by Guazzaroni et al. [35]. Ascorbic acid (1.5 eq.) was added to a solution of Ty (10 mM, 7 mg, 0.05 mmol) in phosphate buffer 0.1 M, pH 7.0 (5 mL). Free tyrosinase (180 µL of a 0.98 mg/mL stock solution in phosphate buffer 0.1 M, pH 7.0) was added and the reaction mixture was stirred at room temperature for 24 h. The aqueous solution was extracted with ethyl acetate, the organic phases were dried over anhydrous Na_2SO_4 and the solvent was evaporated. The crude was analysed by NMR spectroscopy. The obtained conversion was 27%.

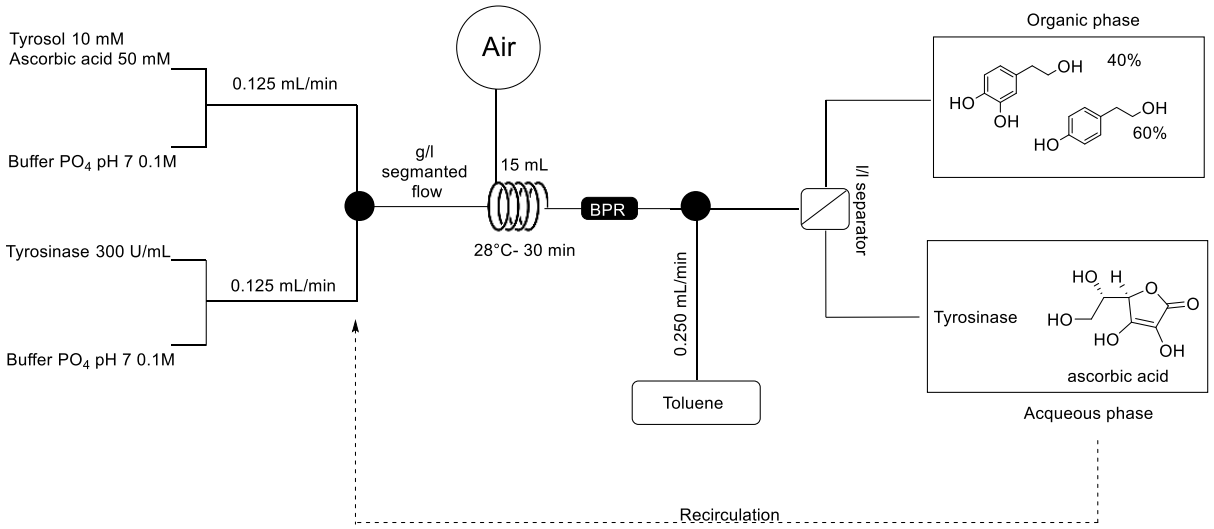
Continuous oxidation of tyrosol by free tyrosinase from *Agaricus bisporus* using a gas/liquid biphasic system and in-line extraction



Scheme 15. Process for the gas/liquid biphasic system

Two stock solutions were prepared: (a) a solution of Ty (10 mM, 14 mg, 0.1 mmol) in sodium phosphate buffer 0.1 M, pH 7.0 (10 mL); (b) a solution of tyrosinase 300 U/mL (360 µL of a 0.98 mg/mL stock solution in phosphate buffer 0.1 M, pH 7.0) and ascorbic acid (50 mM, 88 mg, 0.5 mmol) in sodium phosphate buffer 0.1 M pH 7.0 (10 mL). The stock solutions were pumped through the tubular reactor by two HPLC pumps with a total flow rate of 110 µL/min (55 µL/min for each pump). A third peristaltic pump flowed air at 220 mL/min. The three streams (*i.e.*, the two liquid solutions and the air stream) were mixed in a quadruple mixer to form a gas/liquid segmented flow that entered the coil reactor maintained at 28°C. A 40 psi back-pressure regulator (BPR) was applied to the system (Scheme 4). After the residence time (30 min), the exiting flow stream was extracted in-line by adding an inlet of toluene pumped with an external HPLC pump at 333 µL/min. The outlet flow was directed to a liquid/liquid separator and both organic and aqueous phases were collected. The organic phase was dried with anhydrous Na_2SO_4 and the solvent evaporated. The crude was analysed by NMR spectroscopy. The obtained conversion was 78%. Ty: Rf (DCM/MeOH 9:1): 0.69; ^{1}H NMR (300 MHz, methanol-d_4): 7.05–6.99 (m, 2H), 6.73–6.67 (m, 2H), 3.68 (t, J = 7.2, 0.8 Hz, 2H), 2.71 (t, J = 7.2 Hz, 2H). HTy: Rf (DCM/MeOH 9:1): 0.50; ^{1}H NMR (300 MHz, methanol-d_4): 6.71–6.64 (m, 2H), 6.55–6.50 (m, 1H), 3.67 (t, J = 7.2 Hz, 2H), 2.66 (t, J = 7.2 Hz, 2H).

Continuous oxidation of tyrosol by free tyrosinase from *Agaricus bisporus* using a tube-in-tube reactor and in-line extraction



Scheme 16. Process for tube-in-tube reactor.

Two stock solutions were prepared: (a) a solution of Ty (10 mM, 14 mg, 0.1 mmol) in sodium phosphate buffer 0.1 M, pH 7.0; (b) a solution of tyrosinase 300 U/mL (360 µL of a 0.98 mg/mL stock solution in phosphate buffer 0.1 M, pH 7.0) and ascorbic acid (50 mM, 88 mg, 0.5 mmol) in sodium phosphate buffer 0.1 M, pH 7.0. The stock solutions were pumped at a total flow rate of 250 µL/min (125 µL/min for each pump) (residence time: 60 min) at 28°C. The external tube of the reactor was filled with pressurised air (3 bar) and the whole system was pressurized at 40 psi (Scheme 5). The exiting flow stream was extracted in-line by adding an inlet of toluene pumped with an external HPLC pump at 250 µL/min and then the biphasic flow stream was separated using an in-line liquid/liquid separator. Both organic and aqueous layers were collected. The aqueous phase, which contained the enzyme and ascorbic acid, was recirculated through the system. The organic phase was dried with anhydrous Na_2SO_4 and the solvent was evaporated. The crude was analysed by NMR spectroscopy. The final conversion was 40%.

Catch-and-Release Procedure

The organic flow stream containing Ty and HTy was flowed through a column (total volume: 3.0 mL) packed with polymer-supported boronic acid (1.0 g, loading 2.5–3.0 mmol/g, 1.5 mL) mixed with an equal volume (1.5 mL) of molecular sieves (4 Å) at 110 °C (BPR 1.0 bar) with a residence time of 1 h (flow rate: 50 µL/min). The exiting flow stream was monitored by TLC and only Ty was present. The organic solvent was collected and evaporated under pressure to recover pure Ty.

The release of HTy was performed by flowing 2 N HCl through the column at room temperature with a residence time of 30 min. The solvent was removed under pressure to furnish pure HTy as a yellow oil (75% isolated yield). Rf (DCM/MeOH 9:1): 0.50; ^{1}H NMR (300 MHz, methanol-d4): 6.71–6.64 (m, 2H), 6.55–6.50 (m, 1H), 3.67 (t, *J* = 7.2 Hz, 2H), 2.66 (t, *J* = 7.2 Hz, 2H).

Batch Synthesis of TyAc

To a 0.25 M solution of Ty in phosphate buffer 0.1 M, pH 8.0, 200 mg of imm-MsAcT (1 mg/$g_{agarose}$, 120 U/mg) and 10% v/v of EtOAc (total volume: 1.0 mL) were added. The mixture was left under gentle agitation at 28 °C. After 24 h, the reaction did not reach completion. The reaction mixture was filtered, and the aqueous phase was extracted three times with EtOAc. The organic layers were dried over Na_2SO_4 and the solvent was evaporated. The crude product was purified by flash chromatography (DCM/MeOH 98:2). TyAc was obtained in 47% yield as a white solid.

Flow Synthesis of TyAc and HTyAc and In-Line Work-Up

A glass column (i.d.: 6.6 mm) was packed with 2.5 g of imm-MsAcT (1 mg/$g_{agarose}$; packed bed reactor volume: 2.0 mL). A 0.25 M Ty or HTy solution in phosphate buffer (0.1 M, pH 8.0, with 10% of DMSO in the case of HTy), and EtOAc were mixed in a T-piece and the resulting segmented flow stream (buffer/EtOAc 7:3) was directed into the reactor column that was kept at 28° C. The total flow rate was 200 µL/min (residence time: 10 min). An inlet of EtOAc (flow rate: 200 µL/min) was added to the exiting reaction flow stream using a T-junction and an in-line liquid/liquid separation was performed using a Zaiput liquid/liquid separator. The completion of the reaction was monitored by TLC (DCM/MeOH 9:1). The organic phase was evaporated and purified by flash chromatography (DCM/MeOH 98:2) to yield the desired product. TyAc (yield: 81%): Rf (DCM/MeOH 98:2): 0.56; ^{1}H NMR (300 MHz, $CDCl_3$): 7.10–7.06 (m, 2H), 6.81–6.74 (m, 2H), 4.24 (t, *J* = 7.1 Hz, 2H), 2.86 (t, *J* = 7.1 HZ, 2H), 2.05 (s, 3H). HTyAc (yield: 75%): Rf (DCM/MeOH 98:2): 0.30; ^{1}H NMR (300 MHz, $CDCl_3$): 6.82–6.71 (m, 2H), 6.65–6.55 (m, 1H), 4.23 (t, *J* = 7.2 Hz, 2H), 2.81 (t, *J* = 7.2 Hz, 2H), 2.05 (s, 3H).

Conclusions

HTy is a powerful antioxidant with a number of beneficial biological effects and therefore promises to soon become a staple in natural health care. In this work, an environmentally friendly biocatalysed flow synthesis of HTy using a mushroom tyrosinase and a gas/liquid biphasic system was developed. The precursor Ty is cheaper than both 3,4-dihydroxyphenylacetic acid and oleuropein, which can be used as alternative starting materials for HTy obtainment. The commercial mushroom tyrosinase is rather expensive, but its reutilisation by a closed loop system can reduce the overall process cost. Thanks to this automated, self-sustained setup, HTy was obtained with an isolated yield of 75%, which was much higher than the one obtained in batch. The acetate esters TyAc and HTyAc were then efficiently prepared exploiting a bioreactor packed with the immobilised MsAcT, leading to fast reaction times (10 min), and avoiding enzyme destabilisation for the formation of the ethanol by-product. It is noteworthy that starting from natural substrates, biocatalytic approaches guarantee the commercialization of the final products as natural too.

ii) Whole cells of *Rhodotorula rubra* for the enantioselective reduction of prochiral ketones: development of a tailor-made bioreactor for the continuous production of relevant pharmaceutical intermediates

Article on this topic:

"Continuous-flow stereoselective reduction of prochiral ketones in a whole cell bioreactor with natural deep eutectic solvents"

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Introduction

Biocatalysed redox reactions have received increased attention in the last decades, due to the mild operational conditions and high enzyme selectivity [42]. Alcohol dehydrogenases (ADHs, EC.1.1.1.x) represent a sustainable alternative to non-enzymatic methods for the production of optically active alcohols from prochiral ketones [43–45]. However, this class of biocatalysts relies on expensive cofactors [*e.g.*, NAD(P)+] that require an efficient recycling system usually obtained by coupling a second enzyme (*e.g.*, glucose dehydrogenase and formate dehydrogenase) to achieve an economic and feasible redox transformation. As an alternative, whole cells offer some unique advantages over the use of crude, purified, or immobilised enzyme preparations. First, the presence of native metabolic pathways, as well as endogenous cofactors, can make redox processes self-sufficient. Moreover, lower costs are required for whole-cell catalyst production in comparison with isolated enzymes, which are obtained after, at least in part, cost-intensive purification steps [46–48].

To further reduce the environmental impact of the processes, the use of natural deep eutectic solvents (NADESs) as green co-solvents in enzymatic transformations has recently gained attention [49–52].

In this context, this part of my PhD was focused on the development of bioreductions for the synthesis of enantiopure alcohols, using an immobilised whole cell biocatalyst and an aqueous–NADES mixture in a continuous flow environment [53–55]. In particular, we selected *Rhodotorula rubra*, which has been reported to be a valid biocatalyst for the enantioselective reduction of different substituted β-ketonitriles with good conversion and excellent enantiomeric excess [56], to obtain β-hydroxynitriles, which are important building blocks for the synthesis of different drugs such as fluoxetine, duloxetine, ezetimibe, atorvastatin, and atomoxetine, and selective inhibitors of the serotonin and norepinephrine reuptake (Figure 24) [57].

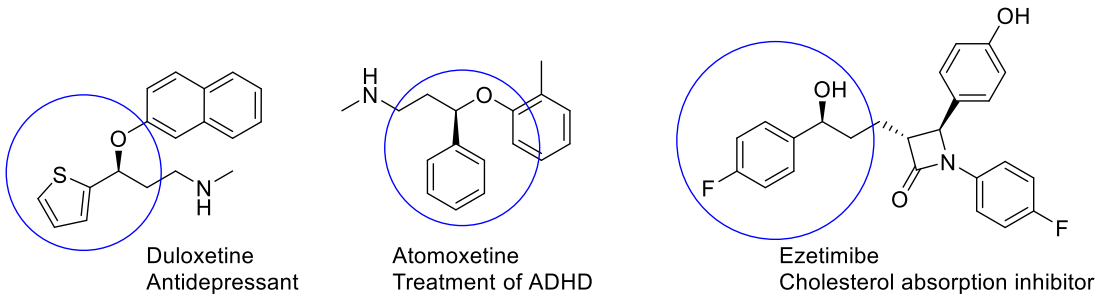
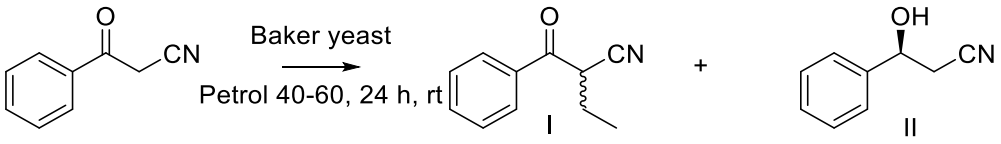


Figure 24. Chemical structure of relevant drugs for which chiral β-hydroxynitriles are key building blocks.

The biocatalyst

Traditionally the enantioselective synthesis of duloxetine has been performed using chiral metal catalysts, usually expensive when not environmentally toxic [58]. The rising interest towards biocatalytic processes for the ecological production of chiral molecules has gained attention for the several advantages connected to this approach and in the last years has been applied to the synthesis of duloxetine. Different microorganisms have been employed for this purpose: *Candida anatrctica*, *Rhodosporodium toruloides*, *Curvalaria lunata* and *Rhodotorula Rubra* [56]. The most common approaches consist in the kinetic resolution of racemic mixture of β-hydroxynitriles mediated by lipases and nitrilases, and in the enantioselective reduction of β-ketonitriles catalysed by ketoreductases. In this way, it was possible to obtain chiral aminoacids as intermediates for the total synthesis of duloxetine with good or excellent enantiomeric excess. However, some limitations are related to these strategies. For example, the kinetic resolution of racemic β-hydroxynitriles mixtures is a combined approach between the biocatalysed transesterification lipase mediated (CalB) and the chemical catalysed racemization by a ruthenium catalyst [59-60]. The use of ketoreductases for the biocatalysed reduction of prochiral ketones depends on the presence of cofactors as NADP/NADPH – NAD/NADH, which are expensive compounds and must be recycled [61]. To prevent this problem the use of whole cells provided the best solution. One example is the employment of *Saccharomyces cerevisiae* to obtain (*S*)-β-hydroxynitriles with excellent enantiomeric excess, but with low yield. This unsatisfactory result is ascribed to the formation of an alkylation by-product. In fact, the cellular metabolism produces acetaldehyde which reacts with the β-ketonitrile through a non-selective secondary reaction of α-alkylation, competitive and non-stereoselective (Scheme 17).



Scheme 17. Reduction of a prochiral ketone with formation of an akylated by-product (I) and an enantiomerically pure alcohol (II).

To avoid this problem, there are some technological options like biphasic systems instead of monophasic ones or lower reaction temperature which allows to shift the reaction equilibrium towards the product [62-63].

In this context, *Rhodotorula rubra* has demonstrated its ability to selective reduce β-ketonitriles with excellent enantiomeric excess without the production of alkylated by-products. This yeast genus is widely spread in nature and has already been employed for the biocatalysed green

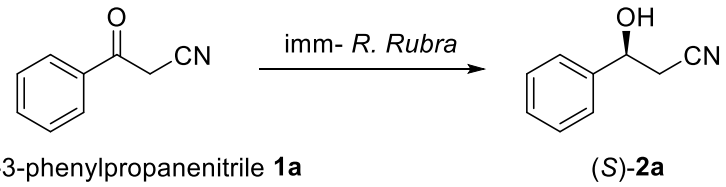
production of chiral pharmaceutical intermediates. Synthesis of chiral 1,3-diols, 1,3-aminoalcohols, 1,3-diamines and 1,3-hydroxyacids could be obtained using different strains of *R. rubra* (MM146 and MM147). Although it is known that *Rhodotorula* species can cause localised infections or fungemia in patients with severe underlying illnesses and compromised host defence, [64-65]they are the object of increasing interest, due to their huge biotechnological potential and several examples of their use for food pharma applications have been reported [66-67].

Results and discussion

R. rubra MIM147 (Figure 25) whole cells were prepared by Prof. R. Gandolfi research group following a slightly modified protocol of one previously reported work [56].

Figure 25. *R. rubra* mucillaginosa colonies.

In view of developing a continuous flow approach, the cells were immobilised. Whole cell immobilisation should keep the cells in a viable state, allowing the stabilisation of their catalytic efficiency and enabling their repeated use. Among the several methods, [68] one of the most used techniques is based on the formation of stable porous gels based on ionotropic gelation of water soluble polyelectrolytes, such as alginate, carrageenan, and chitosan, with oppositely charged ions [69-70]. Therefore, Gandolfi's group firstly immobilised *R. rubra* MIM147 whole cells in alginate beads, a form of immobilisation which has been already proved to be suitable for continuous flow application thanks to good stability over time and after subsequent cycles of biotransformations [38-39], [71]. 3-Oxo-3-phenylpropanenitrile **1a** (Scheme 18) was first used as a model substrate to compare the activity and stability of the free and immobilised biocatalyst in batch mode.



Scheme 18. Reduction of model compound **1a** by immobilised whole cells of *R. rubra*.

Towards this aim, 12 mg mL^{-1} of free cells (dry weight) corresponding to 50 $OD_{530\ nm}$ were suspended in a physiological solution containing glucose (50 mg mL^{-1}) and 2 mg mL^{-1} of substrate, previously solubilised in DMSO (10% v/v). *R. rubra* MIM147 was immobilised in sodium alginate from brown algae (2% w/v) and used in batch biotransformation. After 24 h, the biocatalyst was

recovered and reused under the same conditions. After the first cycle of biotransformation, the molar conversion obtained with the free cells resulted to be much lower than that from the immobilised cells (68% vs. 88%, respectively). After 3 cycles of repeated batch reactions, the conversion with the free cells was less than 10%, whereas the immobilised cells still gave about 55% of molar conversion, indicating that the immobilisation matrix increases the stability of the biocatalyst (Figure 26).

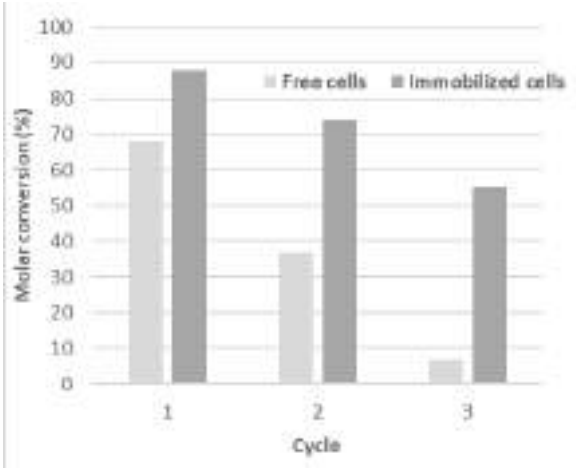


Figure 26. Molar conversions (24 h) obtained with 12 mg/mL of free cells in 2 mL of physiological solution and 12 mg/mL of immobilised cells in 2 mL of 2% w/v alginate from brown algae. Cells were grown in YPD liquid medium for 72 h at 28 °C.

To select the most resistant immobilisation matrix for a continuous flow application and further increase the activity and stability of the biocatalyst, other matrices with different types of sodium alginate were tested:

(i) low density (LD) alginate from *Macrocystis pyrifera*;

(ii) low density (LD) alginate from brown algae;

(iii) medium density (MD) alginate from brown algae.

The biotransformation in each was carried out under the same conditions as reported above immobilizing the cells in 2% of alginate. The molar conversions were similar to those obtained with the matrix prepared with 2% alginate from brown algae (*i.e.*, 28% after 3 h of biotransformation), with the exception of those prepared with alginate from *Macrocystis pyrifera*, which gave a slightly better performance (*i.e.*, 35% after 3 h of biotransformation) and thus was selected. To improve the resistance of the beads, the concentration of the alginate from 2% w/v to 6% w/v was increased. Then, the cells grown for 48 h, 72 h and 96 h in yeast extract peptone dextrose (YPD) liquid

medium were immobilised in the selected matrix. Those grown for 48 h gave the best molar conversion (*i.e.*, 58% after 3 h of biotransformation) and were thus selected for the next batch and continuous flow reactions. A multisimplex approach was exploited for optimisation, considering substrate concentration, buffer pH, and immobilised cell concentration as control parameters [72]; conversion, determined by HPLC, was the response variable. Finally, batch biotransformation was carried out using 26 mg $mL_{alginate}^{-1}$ of cells at 30 °C in 10 mL vial using 1 mg mL^{-1} substrate dissolved in Tris–HCl buffer (0.1 M, pH 7.0, 2 mL) and DMSO (10% v/v) with 2 mL of alginate beads. Glucose (50 mg mL^{-1}) was added to the mixture for cofactor recycling. Optimised batch conditions gave (*S*)-**2a** with 93% molar conversion and e.e. = 99% after 3 h. Notably, as previously reported, no by-product formation was observed [56], [62]. Immobilised *R. rubra* MIM147 cells were reused in repeated batch reduction of compound **1a**, in order to evaluate the operational stability. Towards this aim, the beads were filtered from the reaction mixture, washed with deionized water and reused under the same conditions every 24 h. The molar conversion was evaluated after 3 h of biotransformation. The immobilised biocatalyst resulted to be very stable, being used for 14 reaction cycles, even if a decrease of the reaction rate was observed particularly from the 9th cycle, where conversions after 3 h are below 80% (Figure 27).

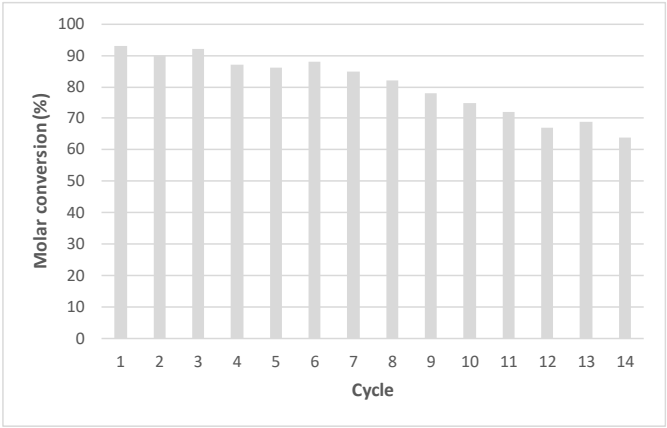


Figure 27. Molar conversions after 3 h of biotransformation in repeated batch with immobilised cells using DMSO as co-solvent

However, it must be noted that full conversion was again achieved after 24 h of biotransformation. In order to avoid the use of DMSO as co-solvent and to increase the sustainability of the reaction, we decided to evaluate the use of a NADES as co-solvent [73-74]. In particular, I selected a choline chloride : glucose (ChCl : Glc, 1.5 : 1 mol/ mol) NADES. ChCl is a popular choice as hydrogen bond acceptor because it is a low cost, non-toxic, biocompatible, and biodegradable reagent which could be produced from natural sources through a very high economy process. On the other hand,

glucose serves both as a hydrogen bond donor and for cofactor regeneration.,[50], [75–78]The molar ratio between choline chloride and glucose was selected in order to have a significant amount of glucose for cofactor recycling without the disadvantage of using a viscous and dense NADES at higher glucose concentrations, which would make it difficult to use in a continuous set-up. The NADES was prepared through a straightforward waste-free process by simply mixing solid ChCl and Glc at 75 °C for 3 h to form a viscous liquid that was used in the batch biotransformations (10% v/v). The use of this less toxic co-solvent, instead of DMSO, also speeds up the biotransformation, enabling a molar conversion of 99% after 3 h. To evaluate the operational stability in the presence of NADES, a repeated batch process was set up (14 cycles), and again the immobilised cells were very stable. For each batch cycle, the substrate solution was replaced with a fresh one, guaranteeing optimal pH conditions (pH = 7.0) and the presence of an adequate amount of glucose for cofactor regeneration. Moved by these promising results, the bioreduction was tested in a continuous flow environment. Freshly prepared alginate beads were packed into a glass column and washed with a Tris–HCl buffer flow stream. However, a constant increase of the pressure was observed during the washing, leading to a partial disintegration of the beads at the bottom and at the top of the reactor. This issue was easily overcome by inserting glass beads at the two ends of the column. Considering the preliminary optimisation performed in batch, I decided to work in Tris–HCl buffer at 30 °C and to evaluate the effects residence time, pressurization and oxygen supply on the conversion using again β-ketonitrile **1a** as the substrate. Oxygen which, in the batch biotransformation, is in the head-space of the vials, takes part in the regeneration of cofactors involved in the biotransformation; therefore, an efficient strategy to guarantee a suitable oxygen supply in the aqueous phase was necessary. First, a solution of compound **1a** (1 mg mL^{-1}) in Tris–HCl buffer (0.1 M, pH 7.0) and ChCl : Glc NADES (10% v/v) was flowed through the packed bed reactor. The residence time was set at 60 min, and I evaluated if the oxygen dissolved in the buffer by bubbling it in the stock solution was enough to guarantee good results. Under these conditions, the biotransformation only reached 40% of conversion. This poor result was ascribed to the lack of available oxygen in the flow stream. So, a segmented gas–liquid flow was applied [79–81]: the gas phase (air, delivered at a constant flow rate through a peristaltic pump) and the liquid phase merged in a T-junction, thus generating air–liquid segments in the flow stream entering the packed bed reactor. The system was pressurised at 20 psi. In this way, the conversion reached 80% in 60 min of residence time. To achieve full conversion, the residence time was further increased to 80 min (Table 21).

Entry	Residence time	Oxygen supply	Pressure	Conversion (%) [a]
1	60	Saturation	Ambient	40
2	60	Biphasic g/l	20 psi	80
3	80	Biphasic g/l	20 psi	99

Table 21. Optimisation of oxygen supply in continuous reactor. Reaction conditions: solution of **1a** (1 mg mL*) in Tris–HCl buffer (0.1 M, pH 7.0) and ChCl : Glc NADES (10% v/v), T = 30 °C. [a] Determined HPLC conversion.

The stability of the system under continuous work was then evaluated, but, unfortunately, the pressure of the system started increasing after 18 h of continuous work, leading to a blockage for overpressure after 24 h (set Pmax = 8 bar). When the immobilised cells were removed from the column, was noticed that a partial breakup of the beads occurred (Figure 28).

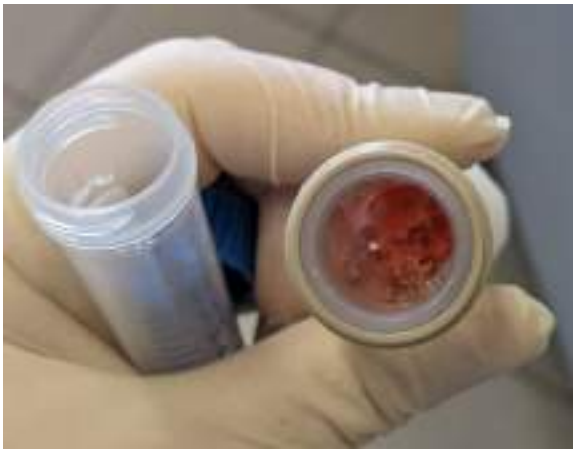


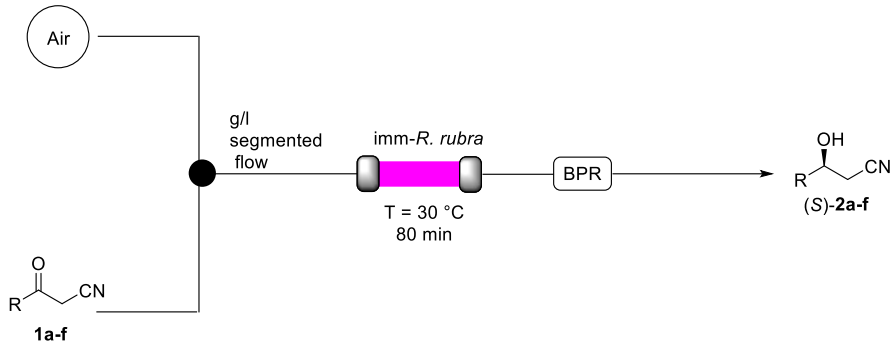
Figure 28. Destroyed alginate beads of R. Rubra by the excessive pressure inside the bioreactor.

For this reason, a different matrix for immobilisation was evaluated. In particular, polyvinyl alcohol (PVA)/alginate mixture was considered as a good option because PVA, which is a non-toxic and cheap synthetic polymer with strong mechanical properties, makes the beads more deformable [82-83]and suitable to better tolerate the pressure inside the reactor. Prof. Gandolfi's group prepared the matrix for immobilisation using 12% w/v and 15% w/v PVA and 1% w/v alginate. The molar conversions obtained after 3 h of batch biotransformation with NADES as co-solvent were comparable and were in the range of 51–54%. Since the matrix prepared with 15% w/v PVA was too viscous and made the immobilisation difficult, the preparation with 12% w/v PVA was chosen, which allowed the obtainment of stable reusable beads with better thermal, mechanical, and chemical stability[84–86]. In order to obtain higher molar conversions, the cell concentration was set at 30 mg mL^{-1}, obtaining a molar conversion of 96% in 3 h of batch biotransformation using substrate **1a**. This immobilisation protocol gave quantitative yields in terms of cell loading into the beads: in fact, after the immobilisation, no activity was found in the supernatant. Moreover, to

determine the activity recovery (AR (%) = activity immobilised cells/activity free cells), the activity of immobilised and free cells under the same batch conditions was evaluated. After 60 min, the obtained AR was 53%. Before moving to flow, the operational stability was tested by reusing the cells every 24 h: up to the 10th cycle of biotransformation, a molar conversion between 96% and 99% was obtained after 3 h, then a 21% reduction in biocatalyst performance was observed at the 14th cycle. The PVA/alginate beads (12 mL) were packed into a glass column and a solution of the substrate, prepared as described above, was passed through it (Figure 29).

Figure 29. Packed bed reactor with *R. rubra* PVA/alginate and PVA beads.

Full conversion of substrate **1a** was obtained again after 80 min of residence time. No increase of the pressure was observed. Specific productivity (SP) under flow conditions was about 1.6 times of the one obtained in batch (SP_f = 0.94 μmol min^{-1} g^{-1} vs. SP_b = 0.60 μmol min^{-1} g^{-1}). Different substrates (**1b–1f**) were then tested, obtaining in all the cases conversions >95% and excellent e.e. (Table 22-Scheme 19). In the case of 3-oxo-3-(thiophen-2-yl)propanenitrile **1f**, whose reduction product is the valuable precursor of the antidepressant drug duloxetine (Figure 24), the system was left running for 120 h. The biocatalyst showed excellent performance; in fact, samples were collected every 12 h and a constant conversion (95%) was observed. After that time, the bioreactor was washed with Tris–HCl buffer, the system was stopped, the cells were recovered from the column and used for a batch biotransformation, obtaining again 97% conversion of substrate **1a** after 3 h of biotransformation.

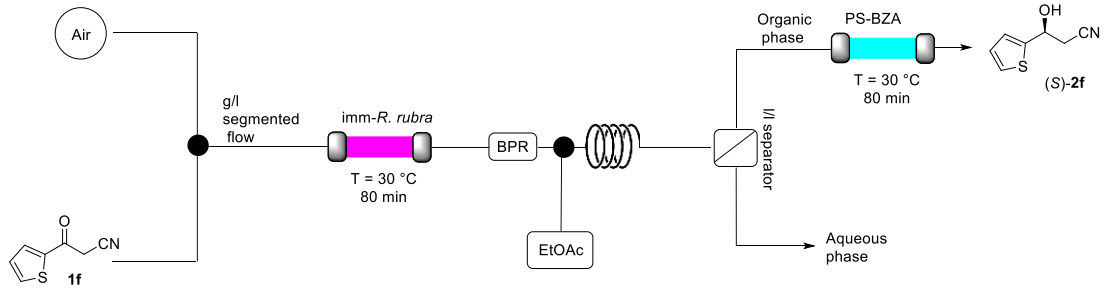


Scheme 19. Continuous set-up for biocatalysed reduction of compound **1a-f**.

Compound	R	m.c. (%)[a]	e.e. (%)[a]
2a	Ph	100	99
2b	*p*-CH_3Ph	100	97
2c	*p*-CF_3Ph	96	97
2d	*p*-OCH_3Ph	100	99
2e	*p*-FPh	97	98
2f	thiophen-2-yl	95	99

Table 22. Molar conversion (m.c.) and enantiomeric excess obtained under optimised conditions. a Determined by HPLC. For details see materials and methods

Under optimised flow conditions, the turnover number was about 2.8 fold the batch one (1.07 $g_{product}$ per g_{cells} vs. 0.38 $g_{product}$ per g_{cells}) and the flow bioreactor productivity (PBR = 12 mL) was 78 $mg_{product}$ day^{-1} [87-88]. Finally, with the aim of developing a fully automated procedure for the synthesis of compound **2f**, an inline purification procedure has been developed (Scheme 20). In fact, continuous integration for *in situ* product removal can significantly contribute to process intensification, together with miniaturization, inline process analytics, and cascade reactions [79], [87–89]. First, an inline liquid/liquid extraction of the desired product was performed adding a flow stream of EtOAc followed by a separation with a Zaiput l/l separator. The organic stream containing the unreacted substrate and the desired product was further purified by flowing it through a column packed with polymer supported benzylamine (PS-BZA), able to trap the ketone **1f** by forming the corresponding imine, allowing the recovery of the pure final product **2f** after simple solvent evaporation.



Scheme 20. Continuous set-up for the synthesis and in-line purification of **2f**.

Materials and methods

Reagents and solvents were obtained from commercial suppliers and were used without further purification. For cell immobilisation, the following alginic acids, purchased from Merck, were used: alginic acid sodium salt from brown algae suitable for immobilisation of microorganisms, alginic acid sodium salt from brown algae low and medium viscosity and alginic acid sodium from *Macrocystis pyrifera* (low viscosity). Continuous flow biotransformation was performed using a R2+/R4 or a Series E Vapourtec flow reactor equipped with an Omnifit® glass column (15 mm i.d. x 150 mm length). The temperature sensor sits on the wall of the reactors. Pressure was controlled using a 20 psi back-pressure regulator. For the inline extraction, an additional HPLC pump (ThalesNano) was used. Inline liquid/liquid separations were performed using a Zaiput separator. MS analyses were performed using a Thermo Finnigan (MA, USA) LCQ Advantage system MS spectrometer with an electron spray ionization source and an 'Ion Trap' mass analyser. The MS spectra were obtained by direct infusion of a sample solution in MeOH under ionization, ESI positive. Biocatalysed reactions were monitored by HPLC analysis with a Merck-Hitachi L-7100 instrument equipped with a detector UV6000LP and reverse phase column (Waters Spherisorb ODS2) or chiral column (OJ-H Chiralcel). ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian Mercury 300 MHz. Chemical shifts (in ppm) were referred to the residual hydrogen/carbon solvent peaks. FTIR spectra were collected with a PerkinElmer (MA, USA) FTIR spectrometer 'Spectrum One' in a spectral region between 4000 and 450 cm^{-1} and analysed by transmittance technique with 32 scansions and 4 cm^{-1} resolution. The turnover number (TTN) is defined as the amount of the product per amount of the biocatalyst over the biocatalyst lifetime.49

Rhodotorula rubra MIM147: culture conditions

Rhodotorula rubra MIM147 was routinely maintained on YPD medium slants (18 g L^{-1} agar, 10 g L^{-1} yeast extract, 10 g L^{-1} peptone, 20 g L^{-1} glucose, pH = 5.6). The strain, grown on YPD medium slants for 72 h at 28 °C, was inoculated in a 100 mL Erlenmeyer flask containing 20 mL of the same liquid medium ($OD_{530\ nm}$ = 2) and incubated on a reciprocal shaker (150 rpm) for 48 h at 28 °C. Two more passages in liquid medium were made over the next few days, leaving the microorganism to grow for 48 h each time. Then, a pre-inoculation was carried out, leaving the microorganism to grow for 24 h and then inoculating it into 50 mL of liquid medium in a 500 mL Erlenmeyer flask (OD_{530nm} = 5). The microorganism was incubated on a reciprocal shaker (150 rpm) for 48 h at 28 °C before testing. Cell growth evaluation. Cell growth was evaluated by UV/Vis analysis, using optical

density as a parameter, and by evaluation of cell's dry weight. Different dilutions of the test sample (1:10, 1:100 and 1:500) were analysed using a spectrophotometer at 530 nm (UV/Vis Jasco V-530). For the evaluation of dry weight, 50 mL of broth culture was centrifuged, the pellet was washed three times with tap water and resuspended in deionized water. The cell suspension was placed in an oven for 12 h at 104 °C.

Preparation of alginate – PVA/alginate matrix

Alginate matrix was obtained by mixing low density sodium alginate from *Microcystis pyriferia* (6% w/v) in deionized water previously heated at 60 °C. For the preparation of the PVA/alginate matrix, PVA (12% w/v) was dissolved in deionized water previously heated at 80 °C under magnetic stirring. Sodium alginate (1% w/v) was then added. The immobilisation solution was then cooled to room temperature.

Immobilisation procedure

Cells obtained by centrifugation (3500 rpm for 5 min) of the culture broth (50 mL) were washed two times with tap water and then resuspended in a certain volume of immobilisation matrix to obtain a desired concentration cells. The mixture was dropped through a 0.7 mm diameter syringe needle into a cross-linking solution containing $CaCl_2$ (2% w/v) under stirring to make alginate beads (30 min) or containing H_3BO_3 (5% w/v) and $CaCl_2$ (2% w/v) under stirring conditions to make PVA/alginate beads (50 min). After washing with deionized water, the PVA/alginate beads were placed in a 0.5 M Na_2SO_4 solution for about 30 min. At the end, the beads were filtered and washed with deionized water before being used (average beads diameter: 2.0–2.2 mm). To rule out a possible negative effect on cell activity of H_3BO, biotransformation with free cells in the immobilisation solution containing H_3BO 5% (w/v) and $CaCl_2$ 2% (w/v) was carried out, obtaining a molar conversion of 93% after 3 h.

Preparation of choline chloride : glucose NADES

A mixture of choline chloride : glucose (molar ratio: 1.5 : 1) was placed in a round-bottomed flask and heated at 75 °C under magnetic stirring until a clear liquid was formed (3 h). The obtained viscous liquid was used in the batch and flow biotransformation.

General procedure for the batch/repeated batch biotransformation using free cells

Fresh cells from sub-merged cultures were centrifuged (3500 rpm for 5 min) and washed with tap water before using. The biotransformation was carried out in 10 mL screw-capped test tubes resuspending *R. rubra* cells in physiological solution (9 g L^{-1} NaCl) at twice the concentration of the submerged culture (12 mg mL^{-1}). Glucose (50 mg mL^{-1}) and the substrate (2 mg mL^{-1}) dissolved in DMSO (10% v/v) were added to 2 mL of the suspension. The reaction mixture was magnetically stirred at 28 °C. In repeated batch biotransformation, the suspension was centrifuged, and the cells washed twice with water. The product was extracted with ethyl acetate (3 times), dried with Na_2SO_4 and the solvent was removed under vacuum. In order to determine the molar conversion of the product, samples were analysed using HPLC Water 600E by Waters Corporation equipped with Waters' Spherisorb ODS2 reverse phase column, eluent: water: MeCN:TFA = 60:40 :0.1, flow rate = 1.0 mL min^{-1} and λ = 210 nm; room temperature; rt: 1a = 8.0 min, 2a = 5.0 min.

General procedure for the batch/repeated batch biotransformation using immobilised cells

A solution of the substrate (1 mg mL^{-1}) was prepared in a mixture of Tris–HCl (0.1 M, pH = 7) and DMSO (10% v/v) and glucose (50 mg mL^{-1}) was added. As an alternative, DMSO and glucose were replaced with the ChCl : Glc NADES (10% v/v). 2 mL of immobilised cells beads were placed in a vial and then 2 mL of the starting solution containing the substrate was added. The mixture was kept under gentle magnetic stirring at 30 °C. After 1 h, 2 h, 3 h and 24 h, 100 μL samples were withdrawn from the reaction mixture, and extracted with ethyl acetate (100 μL). The molar conversion was determined by HPLC. At the end of each cycle, the beads were filtered from the reaction mixture, washed with deionized water and reused under the same conditions. The molar conversion was determined by HPLC.

General procedure for the flow reduction of β-ketonitriles using immobilised whole cells of *R. rubra*

PVA/alginate beads (12 mL) were packed into a glass column (i.d. 15 mm). A stock solution of the β-ketonitrile (1 mg mL^{-1} of substrate in Tris–HCl pH 7.0 0.1 M, 10% v/v NADES) was prepared. The mixture was sonicated (5 min) to completely dissolve the substrate. The solution was pumped using an HPLC pump at 50 μL min^{-1}, mixed with an air inlet delivered using a peristaltic pump at

100 μL min^{-1}, and flowed through the packed bed reactor maintained at 30 °C. A 20 psi back-pressure regulator (BPR) was applied to the system. After the residence time (80 min), a sample of the exiting flow stream was collected and analysed. Molar conversion was determined by reverse phase HPLC analysis (**1a**) or by ^{1}H NMR [90] and e.e. by chiral HPLC analysis. Chiral HPLC conditions: eluent hexane:2-propanol = 90:10, flow = 1 mL min^{-1}, λ = 216 nm; room temperature. Compounds: **2a** rt (*S*) = 24.6 min, (*R*) = 30.9 min; **2b** rt (*S*) = 20.2 min, (*R*) = 22.9 min; **2c** rt (*S*) = 15.3 min, (R) =17.8 min; **2d** rt (*S*) = 40.3 min, (*R*) = 42.2 min; **2e** rt (*S*) =20.4 min, (*R*) = 24.8 min; **2f** rt (*S*) = 28.9 min, (*R*) = 33.0 min.

Synthesis of (*S*)-3-hydroxy-3-(thiophen-2-yl)propanenitrile (*S*)-2f and inline purification

A solution of 3-oxo-3-(thiophen-2-yl)propanenitrile **1f** was prepared (1 mg mL^{-1} in Tris–HCl pH 7 0.1 M, 10% v/v NADES, total volume: 400 mL) and flowed through the packed bed reactor (12 mL, T = 30 °C) as described in the general procedure (flow rate of the substrate solution: 50 μL min^{-1}, air inlet: 100 μL min^{-1}, BPR = 20 psi). The exiting flow stream was extracted inline by adding an inlet of EtOAc pumped with an external HPLC pump at 50 μL min^{-1}. The outlet flow stream was directed to a liquid/liquid separator and the organic was directed into a column packed with polymer supported benzylamine (loading 20 mg g^{-1}, 1 g). The collected organic phase was dried over anhydrous Na_2SO_4 and the solvent evaporated. The desired compound was isolated in 90% yield and 99% e.e. ^{1}H-NMR ($CDCl_3$, 300 MHz, 25 °C): δ = 2.85 (d, *J* = 5.9 Hz, 2H), 5.22–5.30 (m, 1H), 6.95–7.02 (m, 1H), 7.05–7.10 (m, 1H), 7.28–7.32 (m, 1H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): δ = 28.2, 66.2, 117.0, 124.7, 125.8, 127.1, 144. 5 ppm. IR m = 3429, 2922, 2254, 1413, 1040, 850, 709 cm^{-1}. MS (ESI) of C_7H_7NOS (m/z): calc. 153.2, found 175.8 [M^+Na^+]. HPLC analysis: Waters' Spherisorb ODS2 reverse phase column, eluent: water:MeCN:TFA = 60:40:0.1, flow rate = 1.0 mL min^{-1} and λ = 210 nm; room temperature; rt: **1f** = 4.0 min, **2f** = 6.0 min.

Conclusions

The transfer of the catalytic efficiency shown by biocatalysts in nature to chemical processes is a key element to implement the sustainability of a reaction. However, the mere use of enzymes does not provide, per se, a sustainable process. Powerful synergies can be created when environmentally friendly media such as water and deep eutectic solvents are used and when biocatalysis is combined with a continuous flow approach. This combination is particularly attractive for developing greener synthetic protocols, in which biocatalyst stability is increased and the reuse is simplified. In the present work, immobilisation of *R. rubra* whole cells using PVA/alginate beads, beyond the advantages of easier work up and potential catalyst reusability, was found to be an excellent strategy for improving the recyclability of the biocatalyst with respect to the use of cell free systems. The ChCl : Glc NADES resulted to be very efficient as both a co-solvent and efficient system to recycle the cofactor necessary to the reaction and is a greener alternative to DMSO. As further improvement, the enantioselective reduction of β-ketonitriles **1a–f** was performed under continuous-flow conditions exploiting a segmented air–water flow regime, which ensured high mass transfer between the gaseous and the liquid phase. The system resulted to be very stable; in fact, no decrease in the reaction outcome was observed after 5 days of continuous work. Notably, the immobilised whole cells recovered from the packed bed reactor were still highly performing when used in a batch biotransformation. Finally, thanks to a connected software, an automated protocol including an in-line purification procedure was developed for the flow production of (*S*)-3-hydroxy-3-(thiophen-2-yl)propanenitrile **2f**, an intermediate useful for the synthesis of the antidepressant drug duloxetine.

iii) Development of a novel microreactor-based process for continuous biocatalysed synthesis of a biologically active synthetic magnolol derivative mediated by a laccase from *Trametes versicolor*

During my third year of PhD, I spent six months at University of Ljubljana at the Faculty of Chemistry and Chemical Technology (FCCT) working in the Microprocess Engineering Research Group headed by Professor Polona Žnidaršič Plazl and Professor Igor Plazl. Throughout this period, I was able to approach the basic principles of microfluidics and development of tailor-made reactors and reaction systems for intensification of biocatalysed processes, combined with the implementation of deep eutectic solvents.

Introduction

Looking around us, it is outstanding to notice that nature is the best example on how "going smaller" could be advantageous: cells are the perfect example of highly efficient microbioreactors, while capillaries present the optimal microfluidic system for transporting material within higher organisms. However, engineered microfluidic devices have a relatively recent start: the first microanalytical device was developed only in 1975 at the Stanford University (USA) [91]. Advantages of microreactor technologies are related to their small dimensions (by definition in the order of μm or μL), which allow to obtain high surface-to-volume ratio enabling the intensification of all transport phenomena (*e.g.*, mass and heat transport). Moreover, microreactors are associated to better spatial and temporal control which empower a true real time analysis [92]. The first microbioreactor was developed even later, only 28 years ago, for the continuous cultivation of yeast at the Swiss Federal Institute for Technology of Zurich [93]. In recent years, microbioreactor (MBR) systems have experienced a radical evolution towards versatile bioprocess engineering tools. In fact, they offer an inimitable solution to merge higher experimental throughput with extensive bioprocess monitoring and control, which is a crucial point to develop bioproduction processes that could be competitive both ecologically and economically speaking [94]. The trend is clearly visible in Figure 30, where an estimation of the publications present in literature about microbioreactors is reported together with the number of citations [95]. The interest about the topic is rapidly spreading among the scientific community, rubbing off on researchers from all types of backgrounds, from engineers to chemists. Thanks to this wide interest, today is possible to find microbioreactors with different purpose, *i.e.*, organ-on-a-chip systems for the study of the transport phenomena of drugs through biological membranes [96], optofluidic chips for bacteria testing [97], single cell cultivation devices [98], and microbioreactors for biocatalytic processes [88],[99].

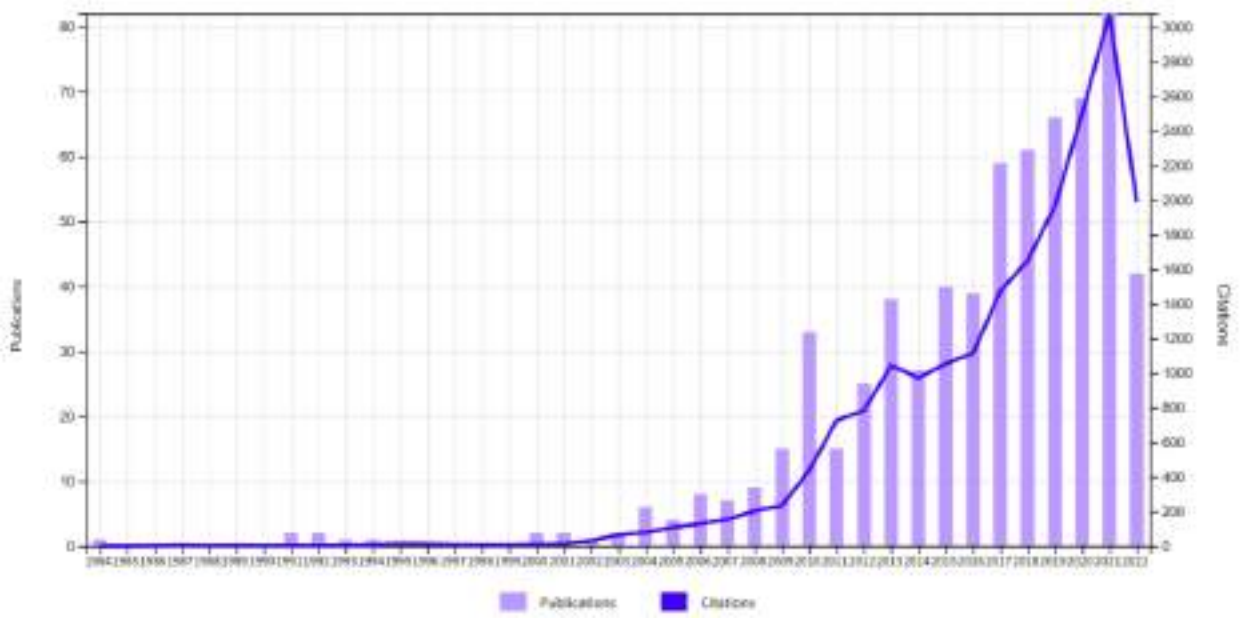


Figure 30. An estimation of the number of publications per year as determined by using "biocatal*"and "microfluid* OR microreact*"as a keyword search in Web of Science (Kindly provided by Prof. Polona Žnidaršič Plazl). [95]

In this context, during my last year of PhD, I decided to explore the possibilities offered by microbioreactors applied to oxidative biocatalysed reactions for the synthesis of nature-inspired biologically active compounds, exploiting the advantages of the micro continuous systems together with the mild operational conditions and high selectivity of oxidoreductases [100].

Magnolia genus has been widely used for centuries in traditional Japanese and Chinese medicine as a treatment for different diseases like anxiety, gastrointestinal disorders, and allergic diseases. After several phytochemical and pharmacological studies to identify the biologically active compounds, at least 255 ingredients were identified. In particular magnolol (Figure 31), a dimeric neolignane, was originally isolated from the bark of Magnolia spp. showing different biological activities like antitumor, antiangiogenic, anti-inflammatory, antimicrobial, antiviral, and antioxidant effects, but also a preventive activity of inflammation-induced tumorigenesis, inhibition of osteoclast differentiation, reduction of multidrug resistance through P-glycoprotein modulation, and protection against cerebral ischemia injury [101-102]. The outstanding number of beneficial effects exerted by this natural compound has resulted in the synthesis and biological evaluation of different analogues. Among them, 1,1′-dityrosol-8,8′-diacetate (**1**) (Figure 31) showed a very potent yeast α-glucosidase inhibitory activity (**1** IC_{50} = 0.15 μM; 98.9% inhibition at 1.5 μM vs magnolol IC_{50} = 2.0 μM and 29.8% inhibition at 1.5 μM) which could be a promising property for the development and optimisation for a new antidiabetic drug.

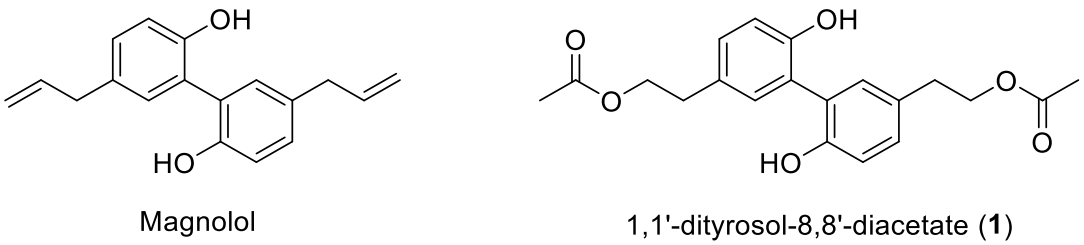


Figure 31. Chemical structure of magnolol and 1,1′-dityrosol-8,8′-diacetate.

Considering its promising properties, a synthetic procedure that allows the obtainment of larger amounts of 1,1′-dityrosol-8,8′-diacetate starting from an easily accessible and inexpensive compound as tyrosol (Ty) appeared highly appealing in the perspective of further biological evaluations. A two-step biocatalysed flow synthesis was designed to obtain the selected compound. The first biocatalyst involved was an immobilised lipase from *Candida antarctica* B packed in a glass column reactor to selectively acetylate the primary alcohol of Ty. The second step is an oxidative dimerisation. H_2O_2-mediated oxidation of simple phenol derivatives catalysed by peroxidases is the most common way to obtain 1,1′-dityrosol-8,8′-diacetate and oxidative products of phenolic compounds, but with generally low yields [102-103]. Considering this limitation, I decided to employ tyrosol acetate (TyAc) as the substrate for laccase from *Trametes versicolor*, a multicopper commercially available oxidase, in order to catalyse the oxidation of TyAc into the desired final product **1**. Since the application of laccase was not previously reported, as far as we know, to produce this dimeric neolignane, a complete characterisation of the product was performed before the thorough study of different continuous systems.

The biocatalyst

Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductases) are multi-copper enzymes that use oxygen in its molecular form to oxidise different aromatic and non-aromatic compounds through a radical-catalysed reaction mechanism [104]. The physiological function of this class of enzymes is to catalyse processes of polymerization and depolymerization. Laccases which are present in different species of fungi are usually involved in lignin degradation, in plants they are responsible for lignification and cell wall formation [105], whereas in insects they are involved in the sclerotization of the cuticle [106]. Their synthetic and degradative role combined to their wide substrate specificity made this class of enzyme an interesting ecological tool for different applications in different fields (*e.g.*, textile, food, paper, pharmaceutical and cosmetics). Moreover, laccases have recently gained wide attention for the one-pot synthesis of complex scaffolds, the selective modification of natural products by oxidation, as well as for the biocatalytic activation of

typically inert Csp^2-H bonds. For this project, a commercially available fungal laccase from *Trametes versicolor* was used to catalyse the oxidative dimerisation of tyrosol acetate (TyAc). Laccases from fungi usually possess three different sites in the copper cluster (Figure 32) depending on their role in the catalytic cycle [107]:

- Type 1 or "blue site" (T1) is involved in substrate oxidation
- Type 2 or "normal site" (T2) catalyses the reduction in molecular oxygen together with T3
- Type 3 or "binuclear site" (T3).

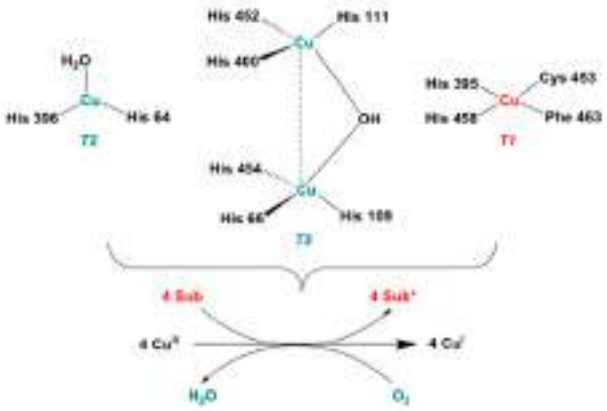
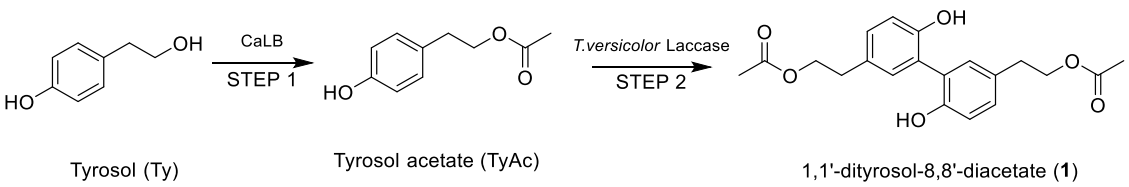


Figure 32. Schematic representation of the active site of the laccase from Trametes versicolor and of the reactions catalysed in a redox cycle [107-108].

Literature reported different ways to employ laccases, based on the redox potential of the substrate of interest: when it possesses the proper redox potential, laccases can be used to directly oxidise it to the corresponding organic radical(s), whereas, when a direct oxidation is not doable due to steric hindrance/active site penetration and/or redox potentials incompatibility, the so-called "chemical mediators" are used with the role of redox intermediates [107].

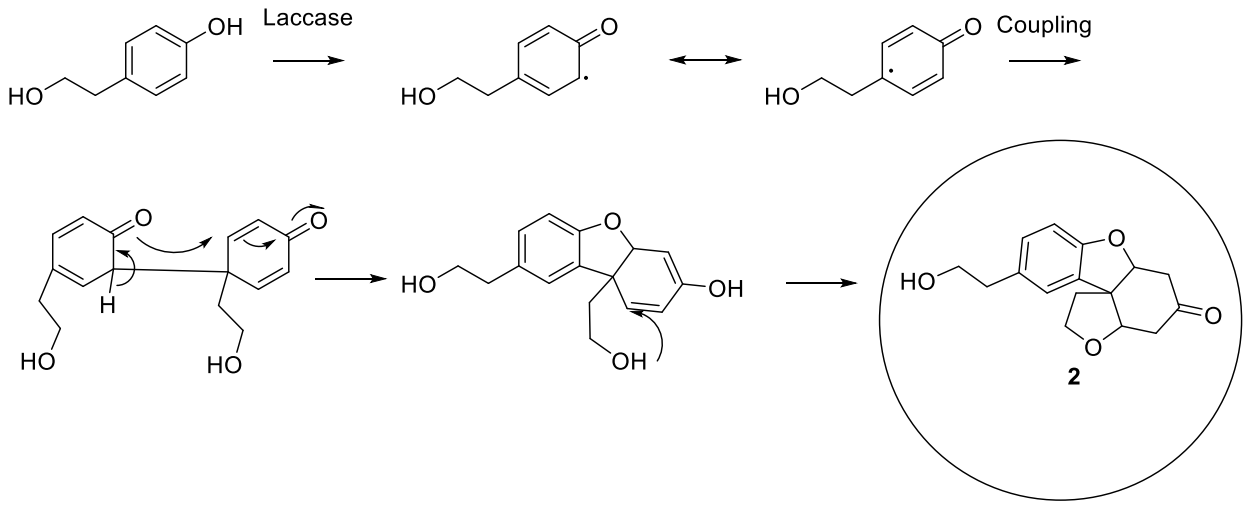
Results and discussion

The biocatalysed synthetic route designed to obtain compound **1** starting from inexpensive Ty rather than from magnolol consisted of two different steps (Scheme 21).



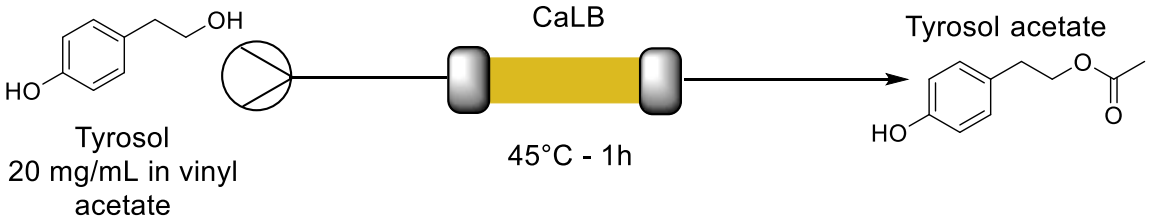
Scheme 21. Two-steps biocatalysed synthesis of **1**.

The first one was the acetylation of the primary alcohol of Ty using a commercially available *Candida antarctica* lipase B (CaLB) immobilised on macroporous acrylic resin, namely Novozym 435® (Novozymes, Bagsværd, Denmark). This step was necessary to mask the primary alcohol and prevent the further reaction of the dimer obtained with Ty that can undergo intramolecular Michael additions resulting in a four-cycle structure compound **2** (produced as racemic mixture as previously reported, Scheme 22) [103].



Scheme 22. Laccase-catalysed oxidation of tyrosol followed by two intramolecular Michael additions resulting in a four-cycle structure, compound **2**.

The acetylation of Ty was performed directly in a continuous system using vinyl acetate both as the solvent/flow stream and acyl donor. A solution of Ty in vinyl acetate was flowed into a packed bed reactor (PBR) containing the biocatalyst and, after a residence time of 1 hour at 45°C, the conversion was completed. Due to the presence of traces of a diacetate by-product, pure tyrosol acetate was recovered after column chromatography with an isolated yield of 92%.

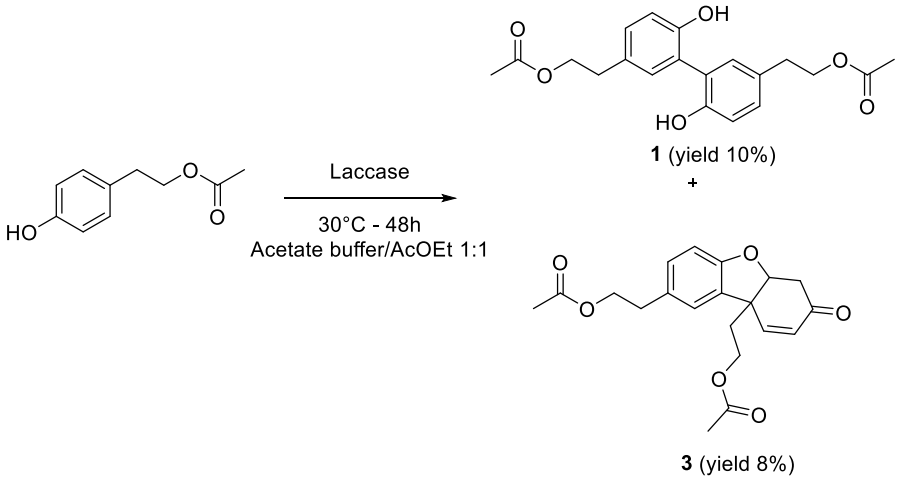


Scheme 23. Lipase-mediated esterification of Ty in flow.

Preliminary studies on the laccase-mediated oxidative dimerisation

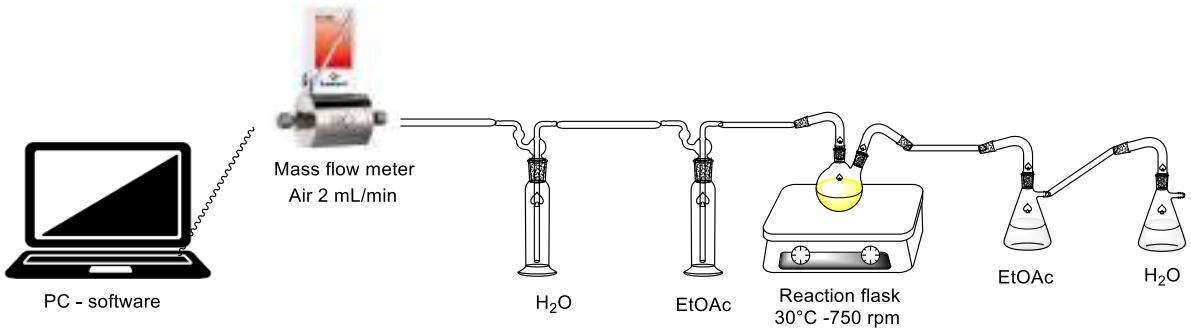
The only literature report on the route to obtain **1** was through the H_2O_2-mediated oxidation of simple phenol derivatives catalysed by horse radish peroxidases as reported by Pulvirenti and

colleagues [102]. However, this route is characterised by low yield (20 %) and requires the addition of H_2O_2. Considering these limitations, I decided to employ tyrosol acetate as a substrate for a commercially available laccase from *Trametes versicolor*, which was not previously reported. Relying on a previous work of Navarra and co-workers [103] concerning the oxidative dimerisation of phenolic compound using the same biocatalyst, the reaction was performed in a biphasic system that allows complete solubilization of tyrosol acetate in ethyl acetate. A batch reaction was performed at 30°C for 48 h in a biphasic system acetate buffer/ethyl acetate (1:1). After purification, a complete characterisation of the isolated compound was done to verify the structure of the final product (*i.e.*, NMR and mass spectra) with the generous help of Prof. Janez Košmrlj (FCCT UL) and his research group. The isolated yield was 10%. Another product (**3**), whose structure is reported in Scheme 24, was isolated (yield 8%) and characterised, and its presence has to be ascribed to a different way of coupling of the radicals species generated by the enzyme. It is relevant to underline the fact that in a batch process, an emulsion difficult to separate was formed due to the mixing and the surfactant properties of the lyophilised preparation of enzyme. This problem could be easily avoided in a continuous system, so this was another rationale to use flow biocatalysis in this process.



Scheme 24. Isolated and characterised products of the batch laccase-mediated oxidation of TyAc.

Once the identities of the product and by-product were confirmed, the influence of oxygen as a second substrate on the reaction was investigated. To perform this in a conventional reaction flask, a system of interconnected vessels was set up and connected as shown in Scheme 5.



Scheme 25. Schematic presentation of a set-up for the investigation of O2 influence on the conversion of Ty into 1.

Using a software-regulated mass flow meter, a constant air flow of 2 mL min^{-1} was provided firstly into a reservoir of water to saturate dry air coming from the central tank with water to prevent aqueous phase removal by exhausting gas. Then, the air flow stream was directed to a second reservoir containing ethyl acetate to saturate the air with the organic solvent and prevent evaporative phenomena in the round bottom flask where the biotransformation was taking place (ethyl acetate/buffer 1:1). After the air was bubbled into the round bottom flask, it was directed into two Erlenmeyer flasks containing ethyl acetate and water to trap potential volatile compounds produced by the reaction. The outcome of the biotransformation with the external aeration was checked through HPLC analysis after 3 and 24 h and compared to the calculated yields obtained with only the oxygen provided by the head-space of the flask. In the reaction, oxygen is reduced by the laccase into water, while the substrate is oxidised into its radical form. For this reason, as shown in Table 23, the presence of external bubbling of air into the reaction medium was a crucial point in order to increase the conversion into the desired product. A continuous supply of air to the reaction system has doubled the conversion after 3 h of reaction time and improved the conversion after 24 h by a factor of 1.64. This was a key point to consider moving into a continuous system since in a closed system, the amount of oxygen would be a limiting factor for the biotransformation.

	Calculated Yield [a] (%)	
Reaction Time (h)	**With aeration**	**No aeration**
3	10.9	5.5
24	28.8	17.6

Table 23. Calculated yield of TyAc with external bubbling of air and without; 10 mg mL^{-1} of TyAc in ethyl acetate, laccase 4-5 U/mL in acetate buffer, 750 rpm, 30 °C. [a] The yield was calculated after the quantification of Ty and Tyac in the crude through HPLC analysis.

Moreover, the impact of the temperature on the reaction rate was evaluated and no relevant variation in the conversion were observed at room temperature (25°C) and at 30°C. In fact, after 24

h with no external aeration the conversion at 30 °C was 17.6 % while at room temperature it was 17.3 %. Therefore, the heating was removed in the following set-ups and the reaction was performed at room temperature.

The next step was the optimisation of the enzyme activity needed for the selected substrate concentration. Different batch reactions were performed in parallel with different enzyme concentrations (1, 2, 4, 6, 8, 10 and 12 U mL^{-1}) and the conversion into the desired product was checked at different times (1, 2, 3, 6, and 24 h) through the HPLC analysis of the organic phase (Figure 33). The calculated yields were than compared and the results shown that 8 units of laccase per mL of buffer solution was the optimum ratio to obtain the highest into compound **1**, 27.96% after 24 h (Figure 34).

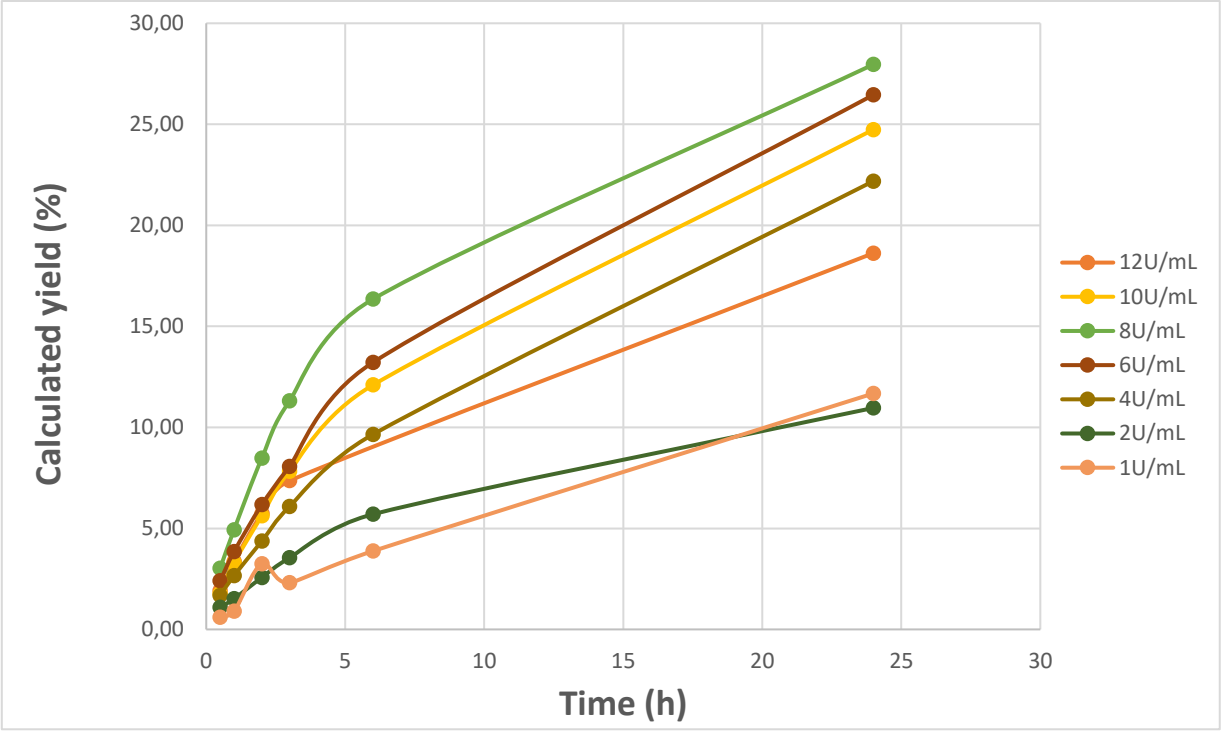


Figure 33. Calculated yield of TyAc with different enzyme concentrations specified in the figure legend. Experimental conditions: Ty Ac 10 mg mL^{-1} in acetate buffer 20 mM pH 3.5, T = 30 °C, 750 rpm.

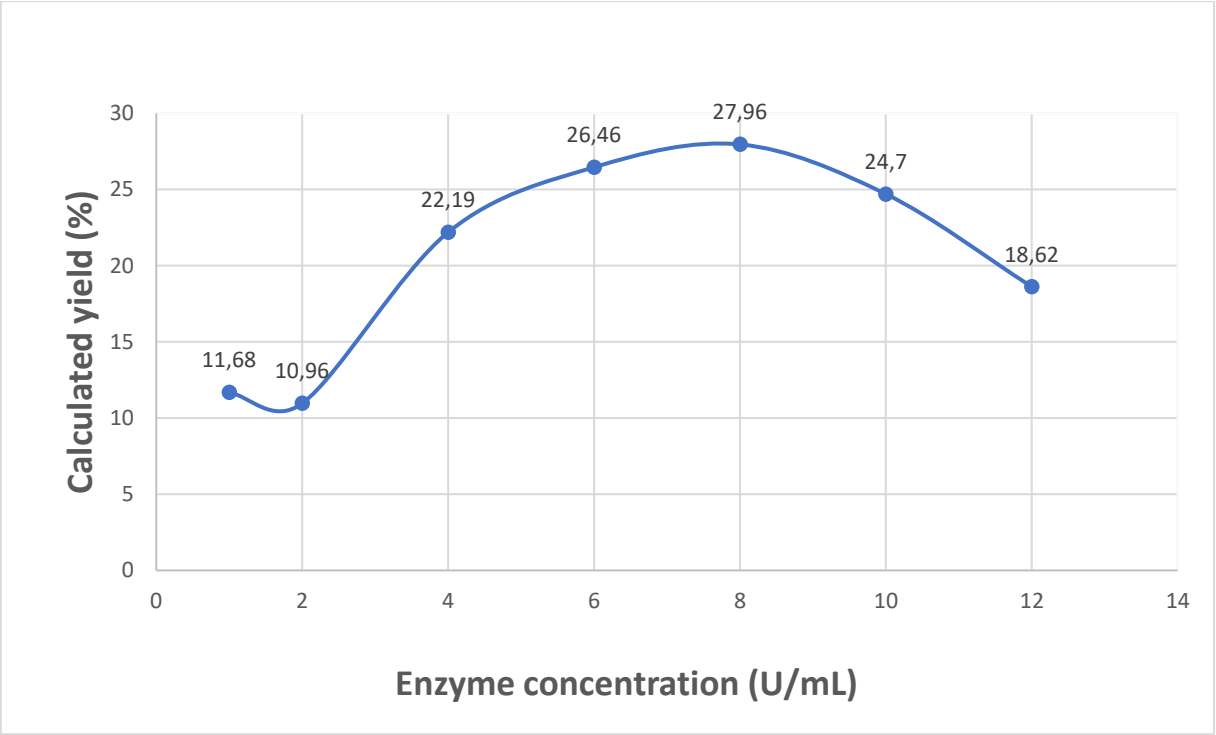


Figure 34. Calculated yield of TyAc with different enzyme concentrations specified in the figure legend. Experimental conditions: batch reaction with Ty Ac 10 mg mL^{-1} in acetate buffer 20 mM pH 3.5, T = 30 °C, 750 rpm, reaction time 24 h.

Continuous reaction with dissolved enzyme in a two-liquid system

Planning to move to the flow, it was necessary to consider that this reaction has challenges such a low solubility of both substrates in aqueous phase, long residence time, and a poorly selective and unstable biocatalyst. To begin the transfer from a batch to the continuous system, a simplified system with enzyme and oxygen dissolved in an aqueous buffer phase, and organic substrate in the ethyl acetate was used. A tubular microreactor that allowed to obtain residence time longer than 2 h was employed assuring detectable calculated yields at the exit. Two syringe pumps were connected through a T-junction and a biphasic slug liquid/liquid flow was obtained. To provide oxygen to the biocatalyst, the aqueous solution was saturated with oxygen. Using flow rates providing 150 min of residence time, the calculated yield was only 1.1%, while at the same reaction time in the batch process performed in a two-liquid system with oxygen dissolved in an aqueous phase, the calculated yield was a few times higher (Table 24). This was a consequence of an additional oxygen available in the headspace of the reaction vial in case of the batch process, confirming the limiting role of the oxygen for this reaction.

To suppress this limitation, a direct inlet of air into the reactor was provided in the next microfluidic set-up. The use of oxygen instead of air was considered and tested but since no significant

variations in the results were observed, the use of air was preferred to reduce the costs and risks of the protocol. The streams of organic phase and buffer solution were joined in a T-junction piece and then mixed with an air flow stream; the resulting liquid/liquid/gas tri-phasic stream was flowed into the microreactor. At the flow rates providing 2.5 h residence time, the calculated yield at the exit of the microreactor was more than 3 times higher compared to the system without a gaseous phase, but still several times lower than in the batch process with the air inlet (Table 24).

		Calculated yield [a] (%)	
Reaction time (h)	**O_2 supply**	**Flow**	**Batch**
2.5	Buffer saturation	1.1	3.8
2.5	Air inlet	3.7	10.9

Table 24. Comparison of calculated yields of compound **1** in a continuous and batch system at room temperature.with various oxygen supplies. TyAc 10 mg mL^{-1} solution in ethyl acetate, laccase 6-12 U mL^{-1} solution in acetate buffer 20 mM pH 3.5. [a] The yields were calculated after the quantification of Ty and Tyac in the crude through HPLC analysis.

The reason for this discrepancy in reaction efficiency was in the fact that the ethyl acetate formed a shell around the bubble of air in a microreactor as it could be seen from Figure 35 presenting a microscope image of the flow in the tube. Therefore, a direct mass transfer of oxygen into the buffer solution where the biocatalyst was dissolved was hindered, which limited the efficiency of the gas/liquid/liquid system in the microreactor compared to the batch system.

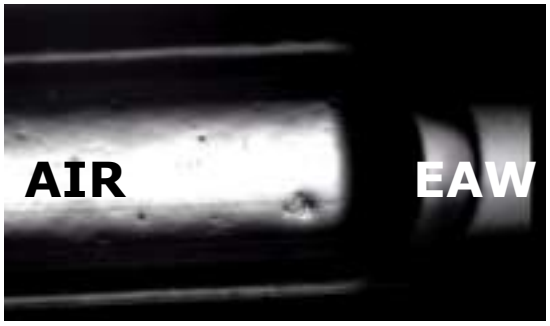


Figure 35. Disposition of a bubble of gas (AIR), the ethyl acetate (EA) and a buffer (W) segment inside the tubular microreactor.

To avoid that, a different combination of the flow streams was considered: the idea was to mix the air and a buffer through a T-piece to have a direct contact between the gaseous phase and the phase where the biocatalyst was dissolved to provide the right amount of O_2 inside the reaction space. Then the organic solution containing the substrate would be added to the system to perform the reaction. However, this ideal liquid/liquid/gas flow was not achievable with syringe pumps and the mass flow controller available due to huge differences of physical properties of the fluids used. To obtain slugs of aqueous solution followed by bubbles of gas it was necessary to use a flow rate of

air significantly higher than the flow rate of buffer, resulting in huge void spaces inside the tubular reactor with small droplets of liquid chaotically distributed. A biphasic gas/aqueous segmented flow regime was achieved using peristaltic pumps, but since their lowest flow rate was 0.1 mL min^{-1}, a reactor volume of more than 30 mL was requested to obtain the appropriate residence times for this reaction. This fact significantly lowered the efficiency of such set-up in an explorative phase.

Medium engineering: deep eutectic solvents as a suitable alternative for ethyl acetate

Considering these preliminary results and the possible denaturising effect of the organic solvent on the biocatalyst, it was clear that proceeding with the further design development of the reactor itself would not be as beneficial as required to make the biotransformation productive as desired. For this reason, medium engineering was the path followed with the essential support of Prof. Marina Cvjetko Bubalo (Faculty of Food Technology and Biotechnology, University of Zagreb) and her research team. Thanks to their collaboration, different deep eutectic solvents were evaluated to replace ethyl acetate as reaction solvent. There are several advantages associated with the use of DESs but the one that was the most attractive in our case was the possibility for DESs to mimic the cell environment which allowed to have a better retained activity of the enzyme [109]. Among the different mixtures tested, two DESs were considered suitable for our reaction system: a hydrophilic DES made by betaine/propylene glycol (Bet/PG molar ratio 1:3) to be used as a cosolvent with buffer for the solubilisation of tyrosol acetate and a hydrophobic one composed of menthol/octanoic acid (Men/Oct molar ratio1:1) to replace the ethyl acetate as organic phase. In order to simplify the system and avoid possible limitation of oxygen transfer to the biocatalyst, the first choice was the hydrophilic DES employed as a co-solvent: 30% v/v of betaine/propylene glycol eutectic mixture was sufficient to obtain a homogenous system where TyAc and the laccase where present. After the mixing of biocatalyst solution and substrate solution, an inlet of air was inserted in order to obtain a segmented flow gas/liquid into the reactor. The calculated yield resulted to be improved (5%) (TyAc 10 mg mL^{-1} in acetate buffer 30 %v/v betaine propylene glycol; laccase 6-12 U mL^{-1} in acetate buffer 20 mM pH 3.5; room temperature; residence time 2.5 h) but not as expected. Moreover, the presence of the co-solvent induced the precipitation of some unknown component of the commercial lyophilised enzyme causing the clogging of the reactor after 3 h of operation. In batch, this phenomenon was visible after just 10 min of reaction. Although this disappointing result, I was able to confirm the necessity to proceed with a triphasic system despite the technological difficulties faced.

The hydrophobic DES selected to replace ethyl acetate was a mixture of menthol and octanoic acid (1:1), a well-known hydrophobic DES with the potential to be used as therapeutic DES [110]. To determine if the replacement of the ethyl acetate was effectively beneficial for the biocatalyst, the original set-up with buffer saturated with oxygen and a segmented liquid/liquid flow was employed. The results obtained using the DES as the substrate solvent phase presented in Table 25 clearly show several fold improvements compared to the use ethyl acetate. This is most likely due to the less harmful conditions for enzyme provided by the DES compared to ethyl acetate which resulted in a better retained activity. However, again the continuous system without gaseous oxygen addition was not as efficient as the reaction performed in a round bottom flask with the head-space filled with air and continuously provided with air (Table 25). Therefore, a direct inlet of air was provided to form a triphasic gas/liquid/liquid system aiming to improve the conversion: compared to the ethyl acetate system, DES increased the conversion by 4.3-fold (Table 25). However, evaluation of a fluid flow regime in a tubular microreactor again revealed the formation of a shell of DES around the bubble of gas, the same as it was observed in the system with ethyl acetate. Likewise, such flow regime hindered direct oxygen transfer between the gaseous phase and a buffer solution.

			Calculated yield [a] (%)	
Reaction time (h)	**O_2 supply**	**Solvent system**	**Flow**	**Batch**
2.5	Buffer saturation	Ethyl acetate/Buffer	1.1	3.7
2.5	Buffer saturation	DES/Buffer	9.5	21.7
2.5	Triphasic g/l/l	Ethyl acetate/Buffer	3.7	n.a.
2.5	Triphasic g/l/l	DES/Buffer	16	n.a

Table 25. Comparison between biphasic system using ethyl acetate and using menthol/octanoic acid s organic solvent. TyAc 10 mg mL^{-1} solution in ethyl acetate or DES, laccase 6-12 U mL^{-1} solution in acetate buffer 20 mM pH 3.5, room temperature. [a] The yields were calculated after the quantification of Ty and Tyac in the crude through HPLC analysis

Considering the improvement driven by the presence of DES in the reaction system, the conversion of TyAc into the final compound was evaluated after 24 h of a batch process with various modes of oxygen supply. The biotransformation was performed in a DES/buffer system (1:1 v/v) both with the buffer saturated with oxygen, and with bubbling of the air in the system as presented in Scheme 25. Results summarized in Table 26 show that the use of DES and buffer saturated with the oxygen increased the conversion after 24 h for more than twice when compared to the ethyl acetate as a solvent for the substrate. On the other hand, the continuous addition of gaseous air to the system did not improve the conversion after 24 h as it was the case with the ethyl acetate, but rather lowered it compared to the one obtained with ethyl acetate or with DES/oxygen-saturated buffer system (Table

26). Moreover, after 48 h, the concentration of product **1** dropped to the half of the one obtained after 24 h.

Reaction time (h)	O_2 supply	Solvent system	Batch Calculated Yield [a] (%)
24	Buffer saturation	Ethyl acetate/Buffer	17.6
24	Buffer saturation	DES/Buffer	40.9
24	Air injection	Ethyl acetate/Buffer	28.8
24	Air injection	DES/Buffer	20.1
48	Air injection	DES/Buffer	10.6

Table 26. Calculated yields of compound **1** after 24 h with different batch systems. TyAc 10 mg mL^{-1} solution in ethyl acetate or DES, laccase 6-12 U mL^{-1} solution in acetate buffer 20 mM pH 3.5, room temperature, 750 rpm. The yields were calculated after the quantification of Ty and TyAc in the crude through HPLC analysis

This phenomenon was explained by the HPLC chromatograms, indicating the formation of a new unknow by-product, meaning that a new species was produced from the further laccase-mediated oxidation of **1**. To verify that, **1** (10 mg mL^{-1} in ethyl acetate) was reacted in the presence of the laccase (6-12 U mL^{-1} in acetate buffer 20 mM pH 3.5) and oxygen coming from the external environment (the round bottom flask was left open), and after 1 hour of a batch process, no trace of **1** was observed, proving that all the starting material was converted into the unidentified by-product. The test of stability of **1** in the reaction mixture without the enzyme revealed that no by-product was formed without the enzyme, confirming laccase-mediated oxidation of **1**.

Reactor engineering: novel microreactor development for gas/liquid/liquid biocatalysed oxidation of tyrosol acetate

The amount and the mode of oxygen supply in the reaction system was found as a crucial parameter for this biocatalytic process. It was found that it is a limiting substrate, but on the other hand its excess in the gaseous form resulted in an over-oxidation into the undesired by-product. Therefore, it was necessary to develop a different microreactor configuration which would provide the biocatalyst in the buffer solution with oxygen along the whole microreactor length, allow for efficient mixing of two liquid phases, and enable the requested residence time. Commercially available reactor for gas/liquid reactions, namely tube-in-tube device was tested, but the air in the external tube acted as an emulsifier for biphasic liquid/liquid flow stream forming a fine emulsion that was not possible to separate (Figure 36).

Figure 36. Emulsion between menthol/octanoic acid and acetate buffer collected at the exit of tube-in-tube reactor.

Thanks to the collaboration of Dr. Drago Resnik from the Faculty of Electrical Engineering of University of Ljubljana, involved together with Prof. Polona Žnidaršič Plazl in the Chair of Micro Process Engineering and Technology - COMPETE, it was possible to develop a suitable device enabling mixing of liquid phases by a passive mixer and oxygen supply *via* polydimethylsiloxane (PDMS).

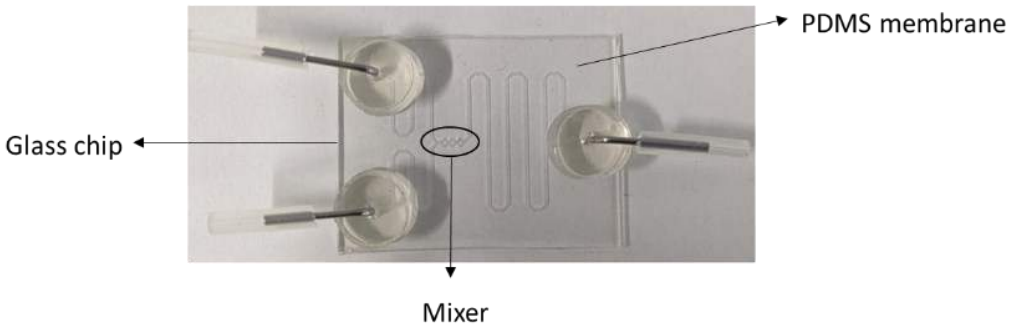


Figure 37. Microreactor for the laccase mediated oxidation provided by Dr Drago Resnik.

The microreactor was made from glass and PDMS where a system of channels was carved (Figure 37). The channels moulded inside the PDMS membrane were covered by the glass chip which acted as a barrier and a solid and clear support for the membrane. Two inlets present at the top of the microreactor enabled the injection of two liquid solutions and one outlet served for the exit of the liquids. Both inlet liquids entered a mixing zone to achieve efficient mixing of both phases, while PDMS layer enabled an indirect contact of liquids with oxygen. PDMS membranes are known to possess several advantages: they are chemical inert, biocompatible, biostable and thermostable, relatively cheap and easy to produce, optically transparent and could be moulded to obtain channel of a few nanometres. Furthermore, they are impermeable to liquids and hydrophobics, but are permeable to gases, in particular the permeability of oxygen through PDMS membranes is well documented in the literature [111].

A solution of laccase in buffer acetate was pumped by means of a syringe pump through one of the inlets at the microreactor, and a TyAc solution in DES menthol/octanoic acid was pumped through the other one. The solution merged at the beginning of the mixer unit and then flowed through the channel with a residence time of 100 min. The microreactor was inserted into a chamber saturated with oxygen. At the beginning of experiments using this device, a hydrophobic DES and an aqueous phase created a sequential slug flow in the glass/PDMS microreactor. After several experiments using the same microreactor, a change in the flow regime to an annular flow with the organic phase as an external layer was observed (Figure 38). This was attributed to the change of PDMS characteristics with time. It is known that after application of plasma treatment for the bonding glass chip with the PDMS, the latter can gain hydrophilic properties for a certain period before turning back to its usual hydrophobicity. Moreover, PDMS is a material which ages with time and could be affected by several uses.

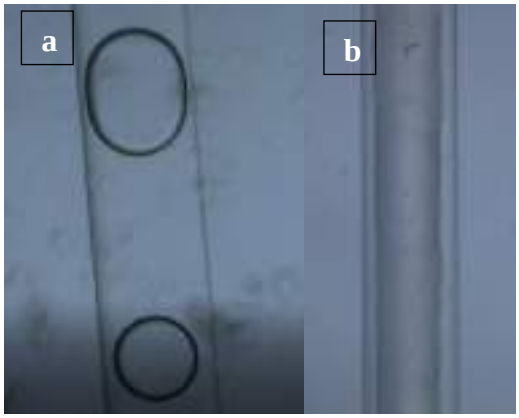


Figure 38. a. Picture of the slugs of aqueous phase inside the DES solution; b. annular stream with the aqueous phase inside and the DES solution on the outside.

The sample was collected at the outlet of the microreactor in a vial placed on ice, where N_2 sparging was used to stop the reaction. The HPLC analysis of the organic phase revealed 22.6 % conversion of TyAc into **1** at the residence time of 100 min, which gave the highest space-time-yield of 1.06 µmol min^{-1} among all tested systems. However, lowering the liquid flow rates to obtain the residence time of 200 min resulted in the clogging of the channels due to the precipitation of some unknow components of the lyophilised preparation of enzyme, as already observed in the monophasic system with the addition of the hydrophilic DES as a co-solvent. This new promising result has to be ascribed to the improved reaction conditions, from the loading of the enzyme (buffer/ DES 2:1 v/v) to the mixing of the liquid phases and the indirect supply of oxygen to the biocatalyst preventing its deactivation.

Materials and methods

Reagents and solvents were obtained from commercial suppliers and were used without further purification. NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer using the residual signal of the deuterated solvent as the internal standard at the University of Milan. NMR spectra at the University of Ljubljana were recorded with a Bruker Avance III 500 MHz NMR spectrometer instrument using the residual signal of the deuterated solvent as the internal standard. ^{1}H chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in hertz (Hz). Mass spectrometry was performed on an Agilent 6224 Accurate Mass ESI-TOF LC/MS system. Continuous flow biotransformations Ty into TyAc was performed using an R2+/R4 Vapourtec flow reactor equipped with an Omnifit® (Merck, Milan, Italy) glass column (6.6 mm i.d. x 100 mm length). The temperature sensor sat on the wall of the reactors. TLC analyses were performed on commercial silica gel 60 F254 aluminium sheets. Continuous flow biotransformations of TyAc in **1** were performed using syringe pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, USA) connected through T-shaped or cross-shaped polyether ether ketone (PEEK) micromixers fluoroethylene propylene (FEP) microtubes (i.d. 794 µm, o.d. 1.58 mm, each tube 8.8 m long) or to a tailor-made microreactor. Gaseous phases were flowed into continuous reactors using Bronkhorst El-Flow mass flow meter/controller (Ruurlo, Netherlands). Biocatalysed reactions were monitored by HPLC analysis with a Varian ProStar HPLC-UV System combined with a direct phase column (Phenomenex Luna 5u Silica (2) 100A, 250x4.6 mm). To monitor the behaviour of the fluid into microreactors a microscope with a high-speed camera (Motion Scope) was used. Laccase from *Trametes versicolor* (≥0.5 U/mg), Novozyme 435®, acetic acid, sodium acetate, ethyl acetate, hexane, *iso*-propanol, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), menthol and octanoic acid were purchased from Merck (Milan, Italy); tyrosol was purchased from Fluorochem (Zentek, Milan, Italy). For laccase from *Trametes versicolor* enzyme activity measurements were performed on a Shimadzu UV-2600 UV-Vis (Kyoto, Japan) spectrophotometer. One unit (U) of activity is defined as the amount of enzyme which catalysis the consumption of 1 µmol of substrate per minute.

HPLC analysis

Direct phase column (Phenomenex Luna 5u Silica (2) 100A, 250x4.6 mm); flow rate 1 mL min^{-1}; λ= 254 nm; mobile phase n-hexane: *iso*-propanol 8:2.

Sample preparation in the case of ethyl acetate as organic phase

Sample 50 µL of ethyl acetate mixture, the solvent was removed flowing nitrogen and the crude dissolved in 50 µL of mobile phase – retention times: tyrosol acetate 4.5 min, **1** 5.5 min.

Sample preparation in the case of Menthol/octanoic acid as organic phase

Sample 10 µL menthol/octanoic acid solution + 40 µL mobile phase - retention times: tyrosol acetate 4.3 min, **1** 5.1 min.

Calculated yields exploiting HPLC calibration curve

Calibration curves were calculated both for tyrosol acetate and 1-1'-dityrosol-8,8'-diacetate and the resulting concentrations of compounds expressed in mg/mL were compared to obtain the calculated yield (([**1**]/[Ty]+[**1**]) *****100).

Continuous synthesis of tyrosol acetate from tyrosol

A glass column (i.d.: 6.6 mm x 100 mm) was packed with Novozyme 435® (1.2 g) to obtain a packed bed reactor (PBR) with a final volume of 3.0 mL. A solution of tyrosol in vinyl acetate (500 mg in 25 mL, 20 mg mL^{-1}) was flowed through the PBR at 50 µL min^{-1} at 45 °C for a total residence time of 60 min. The exiting solution was collected, evaporated under pressure and the crude was purified by flash chromatography (petroleum ether/ethyl acetate 7:3). White solid; yield 92%; Rf = 0.78 (petroleum ether/ethyl acetate 6:4); ^{1}H NMR (300 MHz, $CDCl_3$): 7.10–7.06 (m, 2H), 6.81–6.74 (m, 2H), 4.24 (t, *J* = 7.1 Hz, 2H), 2.86 (t, *J* = 7.1 HZ, 2H), 2.05 (s, 3H).

Laccase activity test

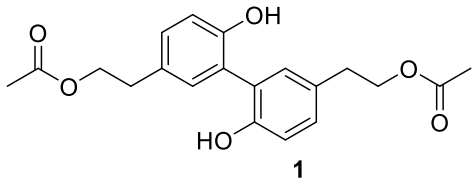
Activity of laccases is determined mostly by photometrical tests using phenolic substrates and by monitoring the coloured oxidation products. ABTS is a common substrate since its oxidation is not pH-dependent between the pH range from 2 to 11 [112]. Determination of laccase activity was carried out spectrophotometrically by monitoring the oxidation of ABTS at 405 nm to $ABTS^{\bullet+}$ (ε_{405} = 36.8 mM^{-1} cm^{-1}).

A 100 µM solution of ABTS in acetate buffer 20 mM pH 3.5 was freshly prepared and stored away from the light together with a solution of 0.25 mg mL^{-1} of laccase in an acetate buffer 20 mM pH 3.5. In a final volume of 1 mL, 25 µL of enzymatic solution, 500 µL of ABTS solution and 475 µL of buffer were mixed and the absorbance change was monitored over a period of 120 s. One unit of enzyme activity was defined as 1 µmol of ABTS oxidised per minute.

Batch oxidation of tyrosol into 1,1′-dityrosol-8,8′-diacetate (1)

In a round bottom flask, 110 mg of laccase (4-5 U mL^{-1}) were dissolved in 10 mL of acetate buffer 20 mM pH 3.5. A solution of tyrosol acetate in ethyl acetate (10 mg mL^{-1}, 100 mg in 10 mL) was slowly added to the solution and stirred at 30 °C. The reaction was monitored by taking samples from the organic phase at different times and further HPLC analysis. After 48 h, the reaction was stopped, the aqueous phase was separated and extracted with ethyl acetate (10 mL x 5). The organic phases were reunited, dried with sodium sulphate anhydrous, and the solvent was removed under pressure. The crude extract was purified through flash chromatography (petroleum ether/acetate gradient).

*1-,1'-dityrosol-8,8'-diacetate (**1**)* [102]

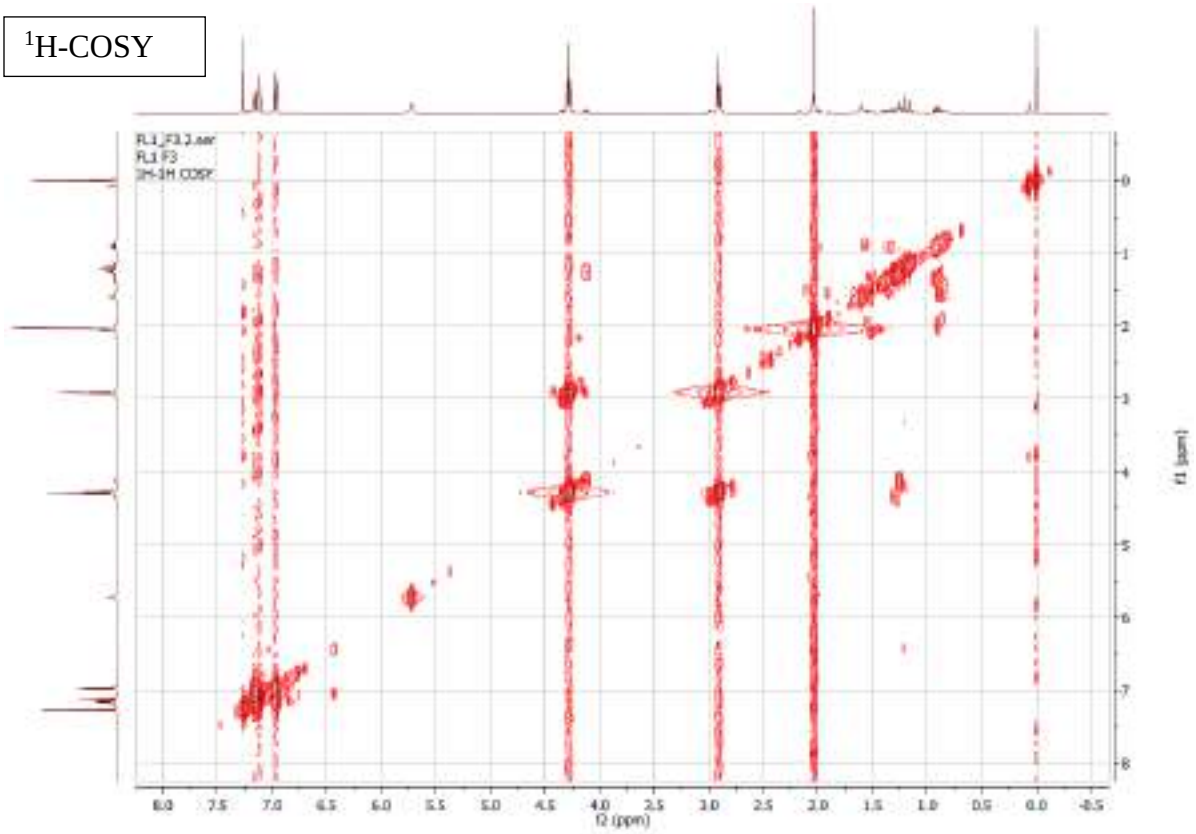


1

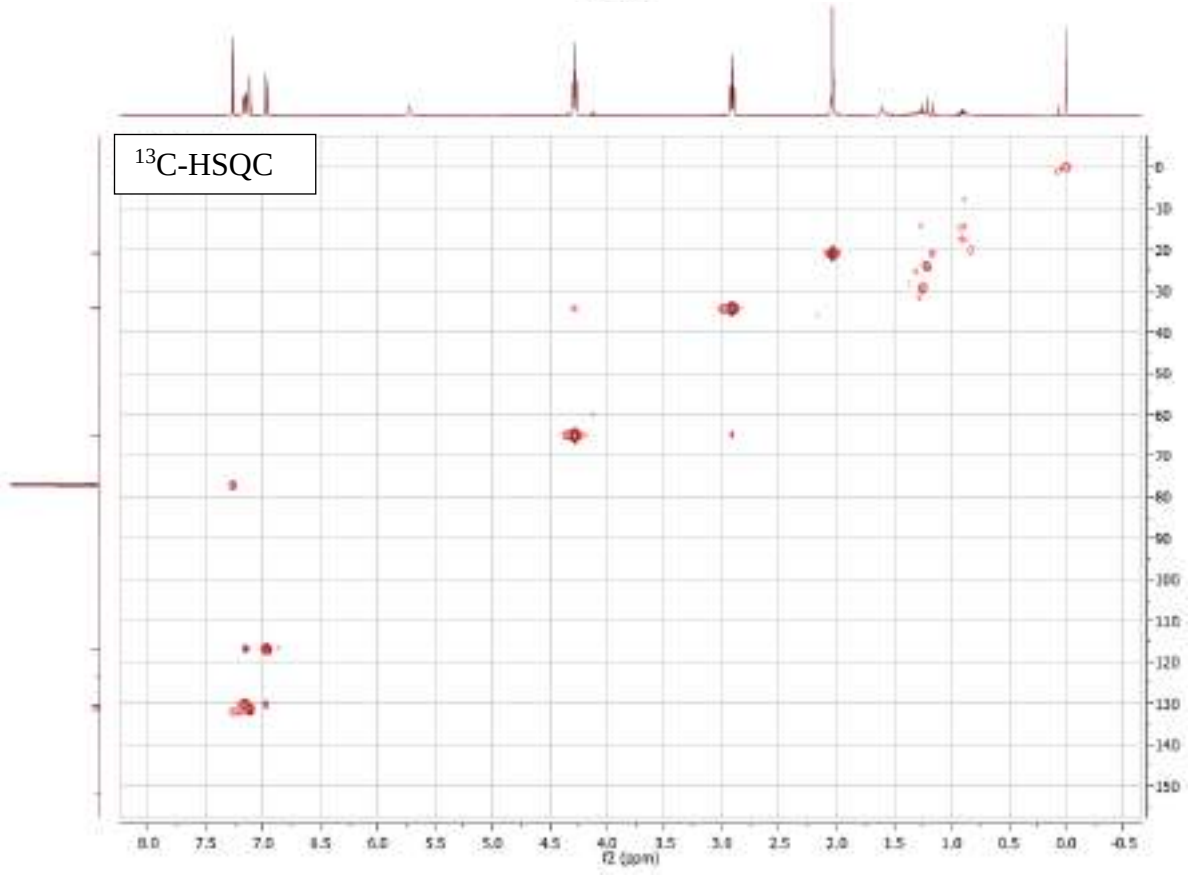
Yellow oil; yield 10%; Rf = 0.3 (petroleum ether/ethyl acetate 1:1), ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.16 (dd, *J* = 8.3, 2.2 Hz, 2H), 7.11 (d, *J* = 2.2 Hz, 2H), 6.97 (d, *J* = 8.3 Hz, 2H), 5.72 (s, 2H), 4.28 (t, *J* = 7.0 Hz, 4H), 2.91 (t, *J* = 7.0 Hz, 4H), 2.03 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.3, 151.6, 131.67, 130.7, 130.7, 123.7, 116.7, 65.0, 34.4, 21.1. MS calculated for $C_{20}H_{22}O_6$ $[M+H]^+$: 359,14; found: 359.14; $[M+Na]^+$ calculated: 381.13; found; 381.13

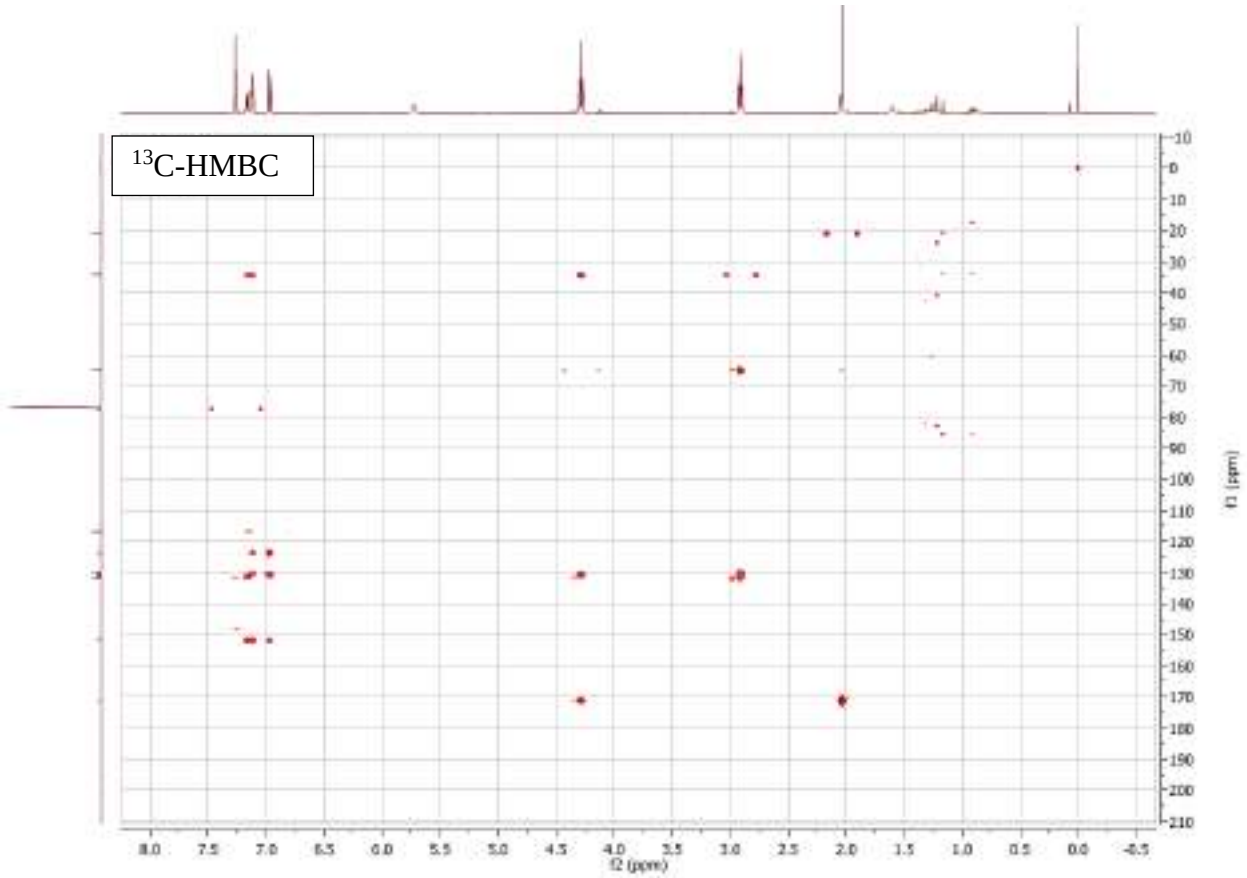
NMR spectra of compound **1**

^{1}H-COSY

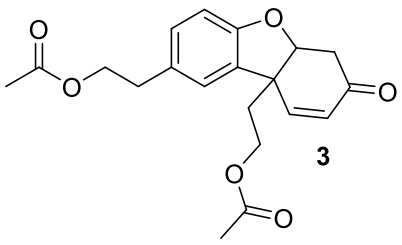


^{13}C-HSQC





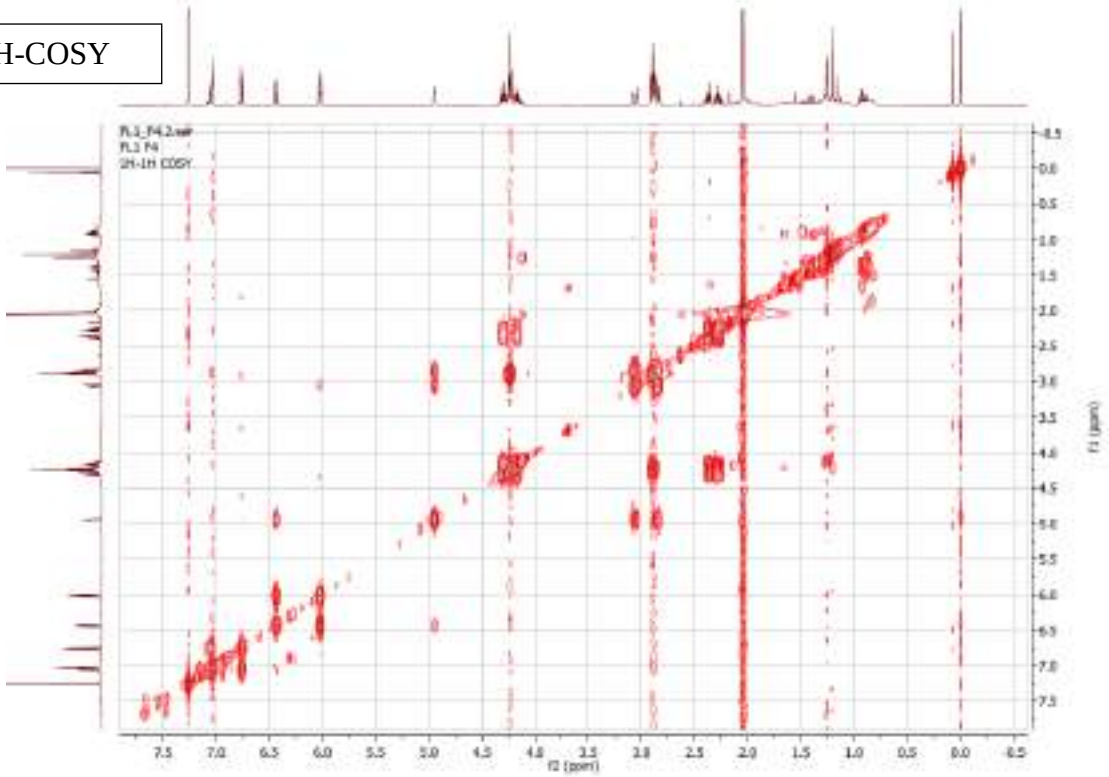
*Compound **3*** [103]



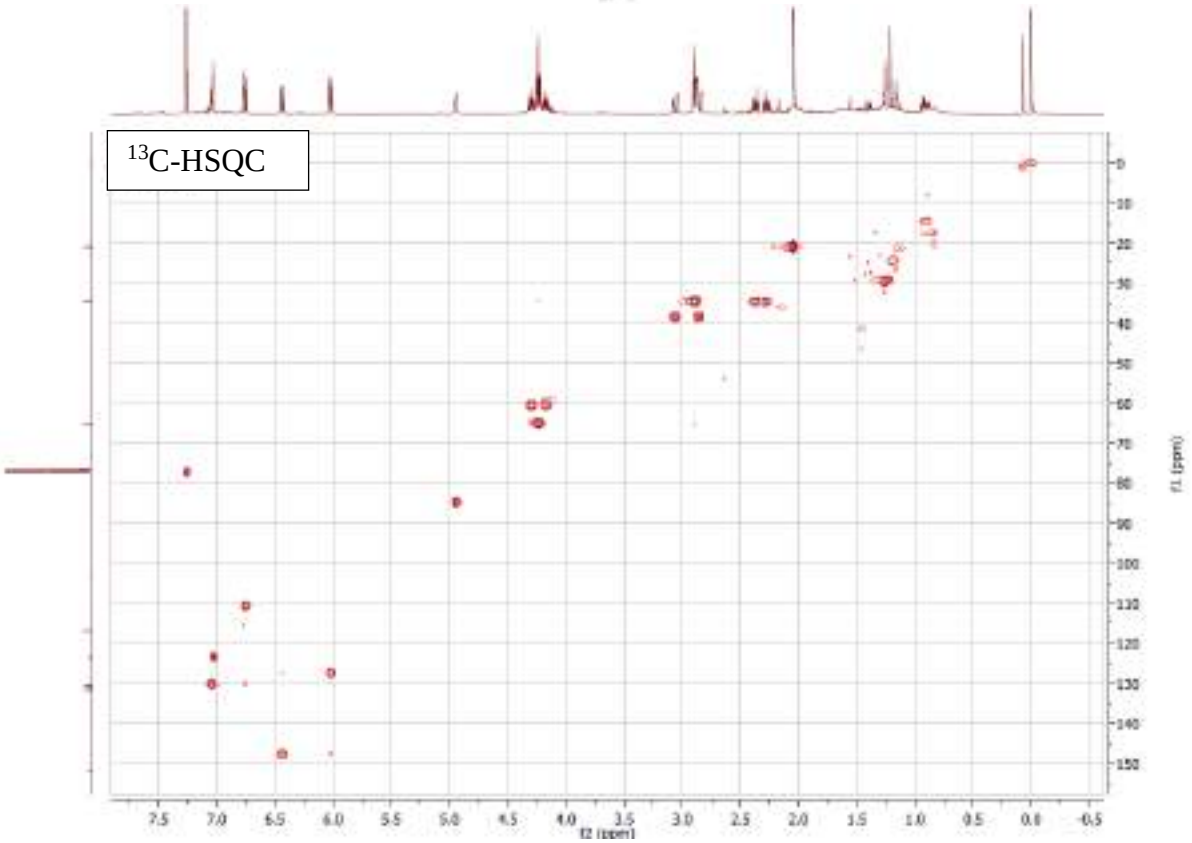
Brown oil; yield 8%; Rf = 0.2 (petroleum ether/ethyl acetate 1:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07 – 6.99 (m, 2H), 6.76 (d, *J* = 8.1 Hz, 1H), 6.44 (dd, *J* = 10.2, 1.9 Hz, 1H), 6.02 (d, *J* = 10.2 Hz, 1H), 4.96 – 4.92 (m, 1H), 4.34 – 4.09 (m, 4H), 3.06 (dd, *J* = 17.6, 2.9 Hz, 1H), 2.87 (ddd, *J* = 17.6, 14.7, 5.6 Hz, 4H), 2.44 – 2.22 (m, 2H), 2.04 (d, *J* = 1.7 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 194.8, 171.0, 170.5, 157.4, 147.6, 131.1, 130.8, 130.1, 127.3, 123.2, 110.5, 84.6, 77.2, 77.0, 76.7, 65.0, 60.5, 47.6, 38.4, 34.6, 20.9. MS calculated for $C_{20}H_{22}O_6$ $[M+H]^+$: 359,14; found: 359.14; $[M+Na]^+$ calculated: 381.13; found: 381.1

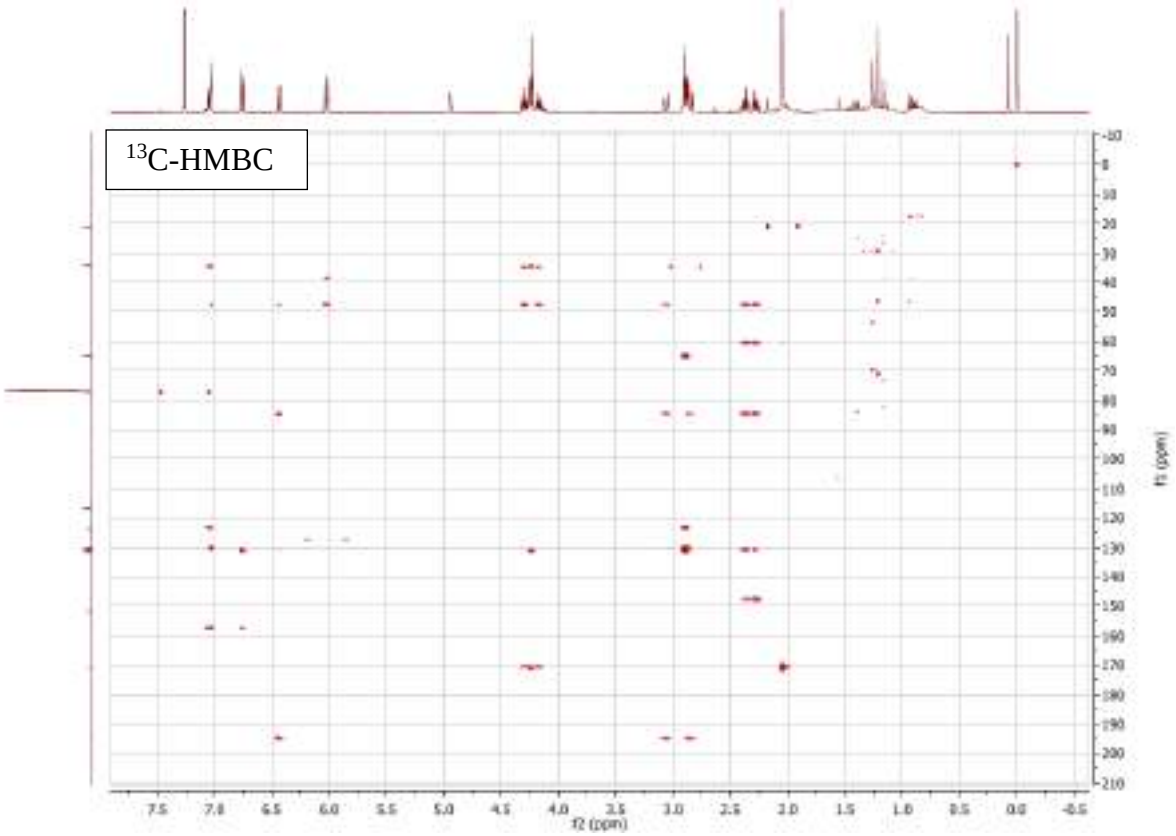
NMR spectra of compound **3**

^{1}H-COSY



^{13}C-HSQC





Menthol/octanoic acid (Ment/ Oct) DES preparation

4.34 g of menthol (1 eq.) were mixed with 4.4 mL of octanoic acid (1 eq.) at 50°C for 15 min. A clear yellow solution was obtained and stored at room temperature.

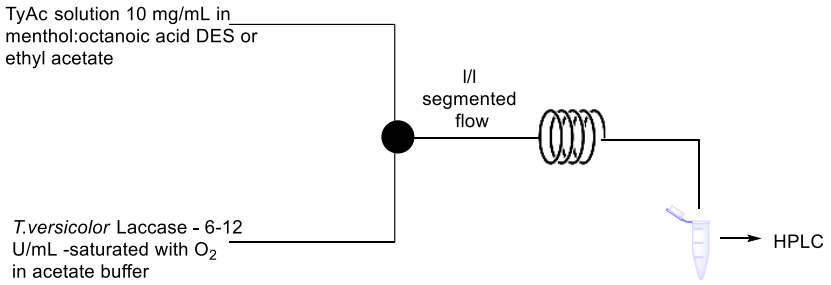
Betaine/propylene glycol (Bet/ PG) DES preparation

4 g of betaine (1 eq.) were mixed with 7.5 mL of propylene glycol (3 eq.) at 50°C for 1 hour. A clear solution was obtained and stored at room temperature.

Continuous oxidation of tyrosol into 1,1′-dityrosol-8,8′-diacetate (**1**) using a liquid/liquid biphasic system

A solution of tyrosol acetate (10 mg mL^{-1}) in the selected organic phase (menthol/octanoic acid 1:1 molar ratio or ethyl acetate) was flowed with a syringe pump at 10 µL min^{-1} into a T-shaped mixer where it was combined with a solution of laccase in acetate buffer 20 mM pH 3.5 (6-12 U mL^{-1}) which was saturated with oxygen for 30 min and pumped with a syringe pump at 10 µL min^{-1}. The segmented flow was directed into a tubular reactor consisting of fluoroethylene propylene (FEP) microtubes (i.d. 794 µm, o.d. 1.58 mm, 8.8 m long) for a total residence time of 150 min (2.5 h) (Scheme 26). The samples at the exit were collected at the steady state using vials placed on ice and

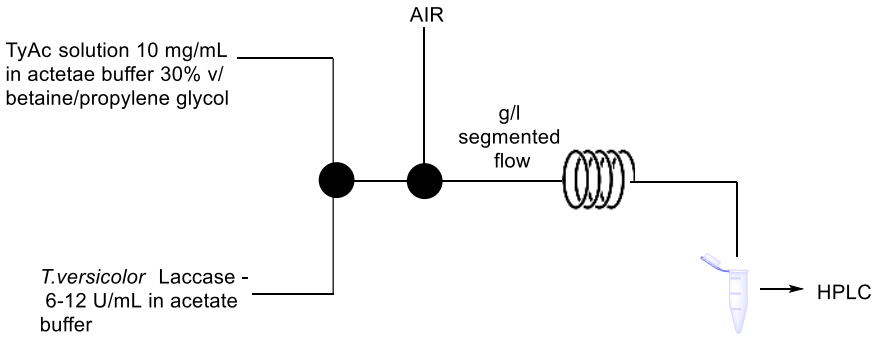
then a sample of organic phase was analysed by HPLC analysis. For the reaction with ethyl acetate, 50 µL were collected, the solvent removed with nitrogen and solubilised in 50 µL of mobile phase; for the reaction with menthol/octanoic acid, 10 µL samples were collected and diluted with 40 µL of mobile phase.



Scheme 26. Continuous set-up for a biphasic liquid/liquid biotransformation.

Continuous oxidation of tyrosol into 1,1′-dityrosol-8,8′-diacetate (**1**) using a gas/liquid biphasic system

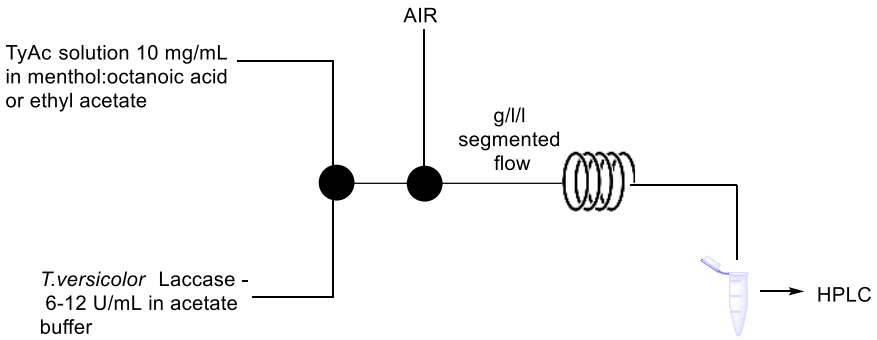
A solution of tyrosol acetate (10 mg mL^{-1}) in acetate buffer 20 mM pH 3.5 30% v/v betaine/propylene glycol (1:3) was flowed with a syringe pump at 10 µL min^{-1} into a T-shaped mixer where it was combined with ta solution of laccase in acetate buffer 20 mM pH 3.5 (6-12 U mL^{-1}). The flow stream was directed into second T-shaped mixer and combined with a flow of air at 20 µL min^{-1} and directed into a tubular reactor consisted of fluoroethylene propylene (FEP) microtubes (i.d. 794 µm, o.d. 1.58 mm, 8.8 m long) for a total residence time of 150 min (2.5 h) (Scheme 27). The samples from the exit were collected at the steady state into vials placed on ice and then a sample (100 µL) was extracted with ethyl acetate (200 µL), the organic phase was separated, the solvent removed, and the crude extract solubilised in 100 µL of mobile phase to be analysed by HPLC.



Scheme 27. Continuous set-up for a biphasic gas/liquid biotransformation.

Continuous oxidation of tyrosol into 1,1′-dityrosol-8,8′-diacetate (**1**) using a gas/liquid/liquid triphasic system

A solution of tyrosol acetate (10 mg mL^{-1}) in the selected organic phase (menthol/octanoic acid 1:1 or ethyl acetate) was flowed with a syringe pump at 20 µL min^{-1} into a T-shaped mixer where it was combined with a solution of laccase in acetate buffer 20 mM pH 3.5 (6-12 U mL^{-1}) at the same flow rate. Using a mass flow controller, the air combined with the segmented liquid/liquid flow through a T-shaped mixer at 40 µL min^{-1} into two tubular reactors made by fluoroethylene propylene (FEP) microtubes (i.d. 794 µm, o.d. 1.58 mm, each 8.8 m long) for a total residence time of 150 min (2.5 h) (Scheme 28). The samples at the exit were collected at the steady state using vials placed on ice and then a sample of organic phase was analysed by HPLC analysis. For the reaction with ethyl acetate 50 µL were collected, the solvent removed with nitrogen and the residue was solubilised in 50 µL of mobile phase; for the reaction with menthol/octanoic acid 10 µL were collected and diluted with 40 µL of mobile phase.



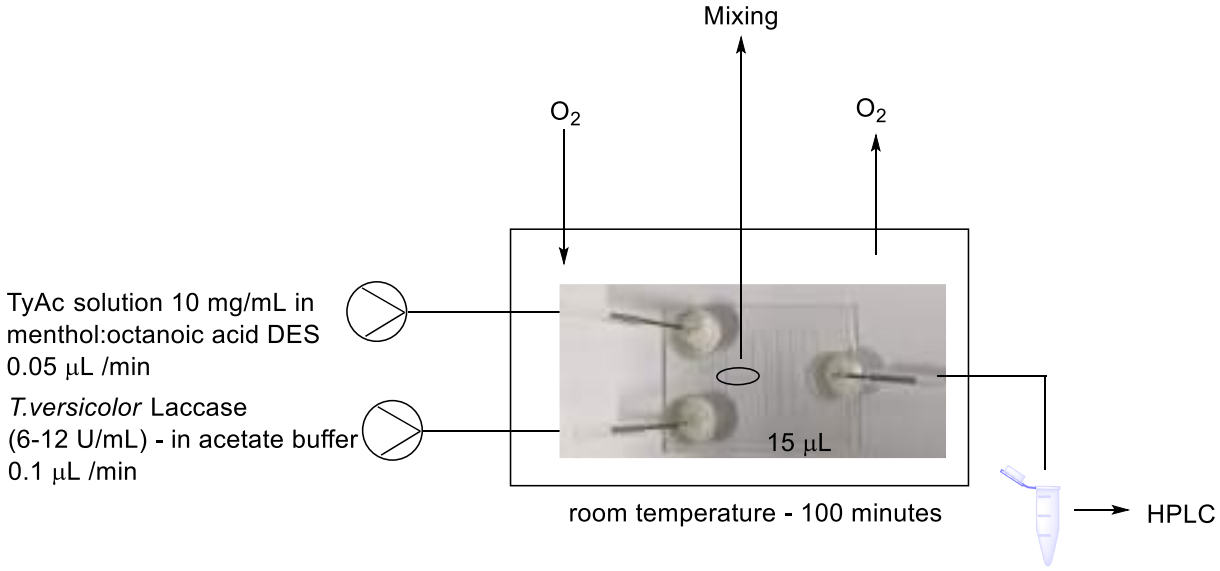
Scheme 28. Continuous set-up for a triphasic gas/liquid/liquid biotransformation.

Laccase mediated oxidation of tyrosol in 1,1’-dityrosol-8,8’-diacetae (**1**) exploiting a commercial tube-in-tube reactor

A solution of tyrosol acetate (10 mg mL^{-1}) in the menthol/octanoic acid (1:1 molar ratio was) flowed with a syringe pump at 50 µL min^{-1} into a T-shaped mixer where it was combined with a solution of laccase in acetate buffer 20 mM pH 3.5 (6-12 U mL^{-1}) at 100 µL min^{-1}. The slug biphasic flow was directed into a tube-in-tube coil reactor (15 mL) where the air in the external tube was pumped with a pressure of 3 bar. The total residence time was of 100 minutes and the reaction took place at room temperature (25 °C). The exiting stream was collected but the biphasic system was completely emulsified.

Continuous oxidation of tyrosol into 1,1′-dityrosol-8,8′-diacetate (**1**) using glass/PDMS microreactor

A solution of laccase in acetate buffer 20 mM pH 3.5 (60 mg in 3 mL of buffer, 6-12 U mL^{-1}) was flowed through the chip at 0.05 µL min^{-1} and a solution of tyrosol acetate 10 mg mL^{-1} (30 mg in 3 mL) in menthol/ octanoic acid (1:1) was pumped at 0.1 µL min^{-1} into the PDMS chip inserted in a closed chamber saturated with oxygen (Scheme 29). The channel after the mixing unit has a total volume of 15 µL. After a total residence time of 100 min, a sample was collected in a vial placed in ice and under inert atmosphere. 10 µL of organic phase were taken and diluted with 40 µL of a mixture of *n*-hexane/*iso*-propanol (8:2) and analysed through HPLC: flow rate 1 mL/min, λ= 254 nm, mobile phase n-hexane: *iso*-propanol 8:2. Retention times: tyrosol acetate 4.3 min, **1** 5.1 min. Conversion: 22.6%.



Scheme 29. Configuration of the continuous reaction with the PDMS chip.

Conclusions

Biocatalytic oxidations and reductions require mild operational conditions and take advantage from the high enzyme selectivity. For this reason, I focused large part of my PhD project on the improvement of continuous redox systems using different biocatalysts. In particular, during my period as a visiting PhD student at University of Ljubljana, I investigated the benefits related to microbioreactors applied to the synthesis of a derivative of magnolol, 1,1′-dityrosol-8,8′-diacetate (**1**), a potential antidiabetic drug, through the oxidative dimerisation of tyrosol acetate. A free laccase from *Trametes versicolor* was selected to catalyse the synthesis of the desired product that, as far as we know, was not reported before using this biocatalyst. After batch studies and a complete characterization of 1,1′-dityrosol-8,8′-diacetate (**1**), the influence of oxygen and the amount of biocatalyst were investigated in batch and different continuous flow systems. Medium engineering comprised the change from ethyl acetate as a solvent to therapeutic deep eutectic solvent composed of menthol and octanoic acid, which resulted to be the best solvent for tyrosol acetate among the tested ones, and also improved the laccase stability and oxygen solubility. The selected green solvent system increased the conversion to over 40% after 24 h, which was 2.3 fold better than with ethyl acetate. However, addition of gaseous oxygen and longer reaction time resulted in overoxidation of **1** into an unknown product. For this reason, a tailor-made microbioreactor made by a PDMS membrane bonded to a glass chip was used to perform the liquid/liquid biphasic reaction supplying the biocatalyst with oxygen from an oxygen-saturated chamber via PDMS membrane. This allowed to reach 22.6% of calculated yield (STY 1.06 μmol min^{-1}) after 100 min of residence time versus 1.1% of calculated yield (STY $0.2 \cdot 10^{-3}$ μmol min^{-1}) of the first continuous process in 150 min. Further optimisation and investigation will be done in the future, comprising immobilisation of the biocatalyst and identification of the unknown by-product.

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CHAPTER 3

Lipase mediated reactions

i) Continuous biocatalysed lipophilizaton of natural phenolic compounds for the synthesis of novel antimicrobial and antioxidant compounds

ii) Lipase mediated continuous synthesis of nature-inspired phenolic carbonate and carbamate derivatives as antimicrobial and antioxidant compounds

i) Continuous biocatalysed lipophilizaton of natural phenolic compounds for the synthesis of novel antimicrobial and antioxidant compounds

Article on this topic:

"Enzymatic continuous-flow preparation of nature-inspired phenolic esters as antiradical and antimicrobial agents"

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Introduction

A large number of phenols and phenolic derivatives occurs in nature, particularly in the plant kingdom, and possess a variety of health-promoting effects such as antioxidant, metal chelator, free radical scavenger, antimicrobial and anti-inflammatory properties [1]. Therefore, plant-derived phenols are of considerable interest from the viewpoint of dietary antioxidant supplementation and food preservation. In fact, food additives are widely used to limit the detrimental effects of food processing and storage. In this context, plant-derived phenolic compounds have great potential as food preservatives [2]. However, due to their hydrophilic character, their applicability as active ingredients in lipophilic food matrices and cosmetic products requiring solubility in non-aqueous media is limited. With the aim to overcome this limitation, common for almost all phenolic compounds, a growing interest has been devoted to synthesising lipophilic derivatives [3]. In fact, the esterification of phenolic compounds for the obtainment of molecules with medium or long aliphatic chains can be used as a strategy for the modification of physical properties, such as solubility and miscibility in emulsion-based systems. Other strategies include the incorporation of one or more halogen atoms [4] or, more commonly, the introduction of different aliphatic chain into the molecular skeleton [5], avoiding the derivatization of the phenolic moiety, to which the biological effects are attributed. Lipophilisation of phenolic acids or alcohols can be performed either following traditional chemical procedures [6], enzymatically [7], or chemo-enzymatically [8-9]. Chemical lipophilisation is commonly achieved under drastic conditions of pH and temperature using strong acidic heterogeneous or homogeneous catalysts resulting in low selectivity with consequent unwanted side products and the need for purification steps that generate extra waste to dispose of. With the evolving demand for green and sustainable processes, the use of biocatalysis has dramatically increased in the last two decades [10-11]. In fact, enzymatic synthesis offers different advantages such as the ability to promote highly chemo-, regio- and stereo-selective transformations under mild reaction conditions, minimization of side reactions and formation of by-products, and more environmentally friendly processes with lower energy consumption and waste production. Enzymatic lipophilisation of phenolic acids or alcohols with hydrophobic chains can be performed in free or added-solvent systems in some hours or several days at temperatures ranging from 30 ° C to 80 ° C [12-13]. Further research should be dedicated to the development of productive and sustainable biocatalytic processes, capable of producing the desired products in sufficient amounts to evaluate their functional, toxicological and sensory profile. In this context, the possibility of merging the advantages of enzymatic catalysis with continuous flow processes can

lead to green, versatile, scalable, and highly productive synthetic tools [14-16]. Exploiting my previous experiences in this field, a simple biocatalysed flow procedure to prepare lipophilic tyrosyl, vanillyl and homo-hydroxytyrosyl alkyl esters was planned to extend their use in pharma, food and cosmetic sectors (Figure 39). In fact, a selective biocatalysed esterification of the primary alcohol of the phenol derivatives with fatty acids (*i.e.*, propionic acid, sorbic acid, caproic acid and lauric acid) was performed to obtain a multifunctional amphiphilic molecule readily available, with a reproducible quality (Figure 39). The scaffolds were selected among natural ones, endowed with interesting antioxidant or antimicrobial properties to achieve a certain variability in the series both on the phenolic and fatty acid moieties [17-19]. Sorbic acid was selected also to evaluate if the presence of the unsaturations conjugated with the carboxylic group could affect the reactivity in comparison with caproic acid and the biological activity of the obtained compounds. Finally, the activity of the obtained compounds as radical scavengers and antimicrobial agents was tested and compared with the parent non-esterified compounds.

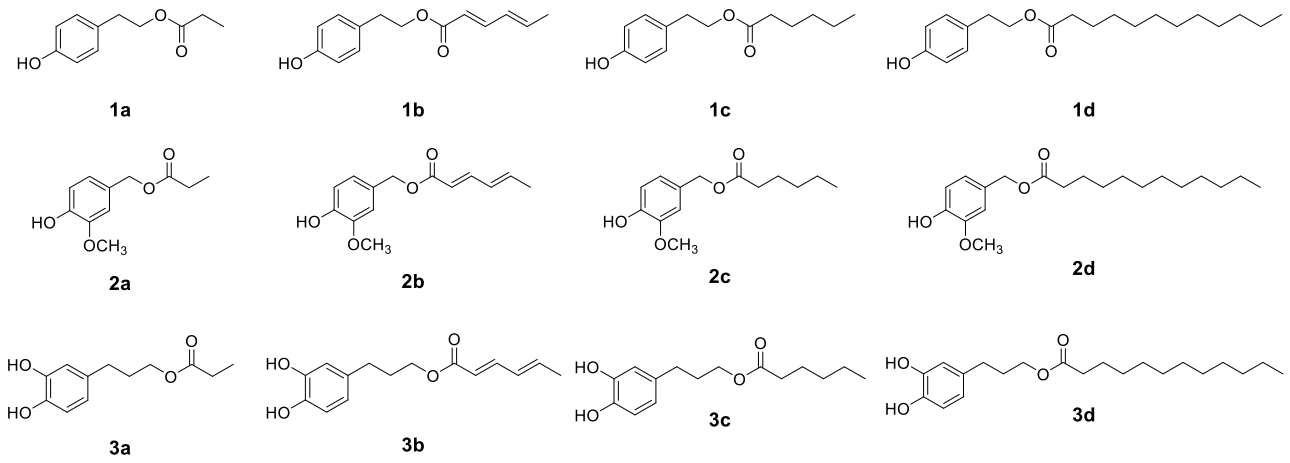
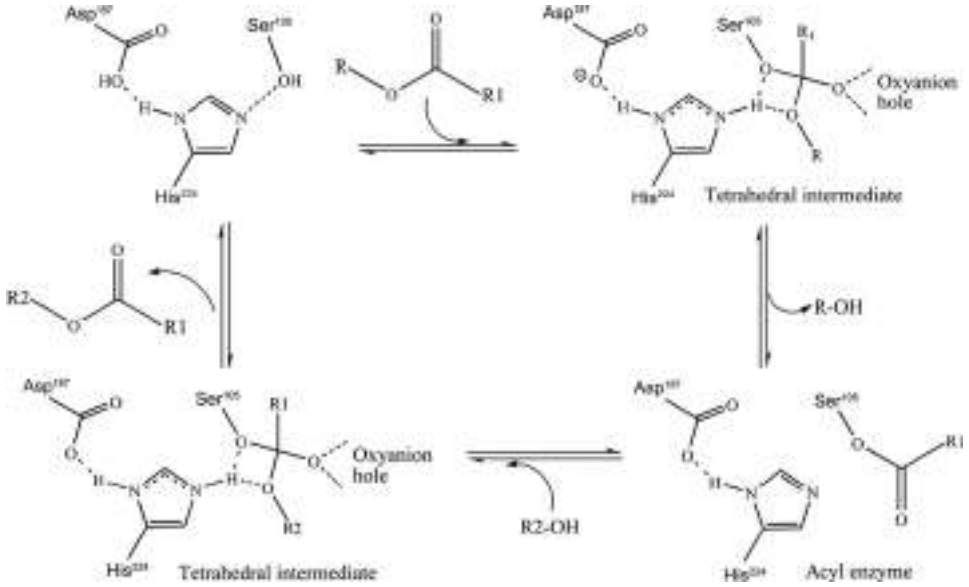


Figure 39. Chemical structure of the synthetised compounds.

The biocatalyst

Lipases are the one of the most used class of enzymes in synthetic organic chemistry, both in academia and industry, since they are able to catalyse the hydrolysis of carboxylic acid esters in aqueous medium or the reverse reaction in organic solvents [20]. Lipase of type B from the yeast *Candida antarctica* (CaLB) is an environmentally friendly biocatalyst employed to produce esters, amines and amides using various enzymatic mechanisms. Its main advantages are its simplicity of use, low cost, commercial availability (also immobilised on polystyrene beads) and recycling, respecting green chemistry principles. Moreover, its immobilised commercial form (Novozyme 435®) has demonstrated to be thermostable at high temperature in organic solvent (up to 100 °C in

diphenyl ether and it retained its catalytic activity when incubated in toluene at 80 °C for up to a month) allowing a wider use of the biocatalyst in different fields from pharmaceuticals, fine chemicals, to agrochemicals production [21]. It is constituted of 317 amino acids and has a molecular mass of 33 kDa. Asp187-His224-Ser105, orientated from left to right, represent the catalytic triad of the enzyme in which the acyl moiety of the substrate lies. Differently, the leaving group or the nucleophile moiety lay in the medium hydrophobic pocket [22]. Immobilised CaLB is widely used packed in glass column as a versatile bioreactor.



Scheme 30. CaLB mechanisms of hydrolysis and transesterification [23].

Results and discussion

Since the quest for green solvents derived from renewable sources in biocatalytic reactions is currently matter of intense research[24-27], cyclopentyl methyl ether (CPME) was chosen as the solvent for the biotransformation [28-29]. CPME is an aprotic solvent employed in organic chemistry as multipurpose solvent (extractions, polymerizations, crystallisations, and reaction media). It is currently synthesised by petrochemical-based routes with high atom economy, but some bio-based alternatives are becoming available, paving the way for a future biogenic source of the solvent, potentially minimizing waste production and energy consumption. CPME has become a greener alternative to extensively used solvents such as tetrahydrofuran, *tert*-butyl methyl ether (TBME) or 1,4-dioxane. In fact, it can be employed in a similar way but with more safety due to its high boiling point (106 °C) compared with other ethers, and its low tendency to generate peroxides. CPME shows high hydrophobicity resulting to be easy to dry, stable under acidic and basic conditions, oxidants, reductants, organometallic reagents, nucleophiles, etc [30]. Moreover, CPME is safe thanks to of the lack of mutagenic and genotoxic activities in *in vitro* test [31].

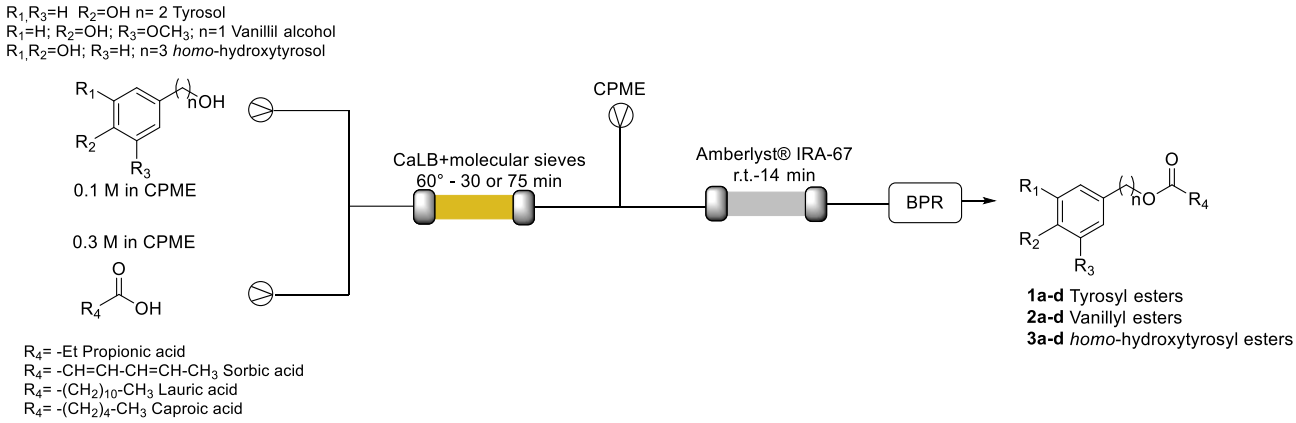
The reaction using tyrosol (Ty) and lauric acid as the substrates was studied to optimise the reaction parameters, including the molar ratio of the substrates (1:1, 1:2, 1:3), concentration (range: 0.02-0.1 M), temperature (range: 40-80 °C), residence time (range: 7-30 min), and stability under continuous work (Table 27). The conversion was monitored by HPLC. A packed bed reactor (PBR) was prepared mixing the immobilised *Candida antarctica* lipase B (CaLB) with molecular sieves (4 Å, 1:1 w/w). After a first set of experiments, a 1:2 ratio between Ty (0.1 M in CPME) and lauric acid (0.2 M in CPME) was selected. Then, the effect of temperature was investigated. Different temperatures between 40 °C and 80 °C were tested. At 80 °C, the conversion remained constant as at 60 °C, reaching 84% conversion in 15 min of residence time. Thus, the optimal temperature of 60 °C was identified. By increasing the equivalents of lauric acid to 3 eq., full conversion was achieved (residence time: 15 min, T = 60 °C, SP_f = 29 μmol $min^{-1}g^{-1}$) [32-33], thus, these were identified as the best conditions. Under batch conditions, the conversion was 60% after 24 h (Table 27).

Entry	[Ty] (M)	[lauric acid] (M)	Residence Time (min)	T (°C)	Conversion [a] (%)
1	0.02	0.02	15	40	82
2	0.05	0.05	15	40	62
3	0.1	0.1	15	40	48
4	0.1	0.2	15	40	65
5	0.1	0.2	15	60	84
6	0.1	0.2	15	70	85
7	0.1	0.2	15	80	82
8	0.1	0.2	30	60	92
9	0.1	0.3	15	60	>99
10	0.1	0.3	7	60	92
11	0.2	0.6	15	60	71
12	0.2	0.6	30	60	>99
batch[b]	0.05	0.15	24	60	60

Table 27 Biocatalysed flow synthesis of compound **1d**: screening of the reaction conditions. Experimental conditions: packed bed reactor: 3.0 mL, BPR: 75 psi. A solution of Ty (0.05 M) and lauric acid (0.15 M) in CPME (20 mL) was prepared. Immobilised lipase from *Candida antarctica* (312 mg) and molecular sieves 4 Å (312 mg) were added. The solution was stirred at 60 °C for 24 h and monitored by HPLC.[a] Determined by HPLC. Conversions (%) are the average of two experiments.

The higher conversion under flow conditions can be attributed to the better heat and mass transfer, absence of back mixing and no shear stress on immobilised lipase due to agitation. Higher enzyme loading can be employed to increase the performance in batch reactor, but this would not be convenient in terms of economy. A time on stream study was performed to evaluate the stability of the catalyst in an unconventional medium over a prolonged time. In particular, the production of 4-hydroxyphenethyl dodecanoate **1d** was monitored under continuous work carrying out the biotransformation for 24 h under optimised conditions (residence time: 15 min, T = 60 °C, back pressure regulator: 75 psi, 3 eq. of lauric acid, flow bioreactor productivity = 4.1 $g_{pruduct}$/day) and monitoring the exiting flow stream by HPLC. The bioreactor showed excellent stability, obtaining constant conversion (> 99%) during the monitoring. Then, the catalytic bed was rinsed with CPME at 0.5 mL min^{-1} for 15 min. The bioreactor was then used for the second cycle of reaction under the same conditions. Similarly, ten set of cycles were repeated. The results show again almost full conversion (98%) at each cycle, proving the reusability of the enzyme. Moreover, with the aim of developing an automated procedure for the synthesis of the desired esters, an in-line purification procedure was developed by adding a column packed with Amberlite®IRA-67 to scavenge the excess of the carboxylic acid (Scheme 31) at room temperature (25 °C), that can be released and recovered by flowing through the scavenger column a 2% v/v solution of acetic acid in CPME.

Exploiting the final set up, all the substrates were submitted to the esterification reaction and the products were isolated in good to excellent yields (*i.e.*, 59-90%). However, the reactions using sorbic acid appeared more challenging, giving very low conversions (5-10%), probably due to the presence of double bonds conjugated to the carboxylic group that negatively affects the lipase activity. Therefore, in this case, the residence time was increased to 75 min to isolate the desired esters **1b, 2b** and **3b** in acceptable yields (24-30%). A further increase of the residence time (*i.e.*, 90 min and 120 min), of the temperature (*i.e.*, 70 °C and 80 °C) or of the equivalents of sorbic acid was not beneficial, leading to similar conversions and productivities.



Scheme 31. Continuous synthesis and in-line purification for the synthesis of different esters of natural phenols.

Since phenolic compounds can act as free radical scavengers due to their ability to donate a hydrogen radical forming aryloxy radicals, their efficiency as radical scavengers was evaluated performing a 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging assay [34]. The results are summarised in Table 28.

Compound	(%) Inhibition
Ty (0.1 mM)	45±1
1a (0.1 mM)	32±2
1b (0.1 mM)	38±0.5
1c (0.1 mM)	22±2
1d (0.1 mM)	35±4
VA (0.1 mM)	51±3
2a (0.1 mM)	26±2
2b (0.1 mM)	40±2
2c (0.1 mM)	3±1
2d (0.1 mM)	33±2
***Homo*-HTy** (0.01 mM)	35±1
3a (0.01 mM)	50±2
3b (0.01 mM)	62±4
3c (0.01 mM)	40±1
3d (0.01 mM)	52±2

Table 28. Determination of the free radical scavenging capacity. Ty: tyrosol; VA: vanillic alcohol; homo-HTy: homo-hydroxytyrosol.

The structures of the aromatic alcohol as well as of the carboxylic acid used for the ester formation significantly affect the radical scavenger properties of the obtained compounds. As expected, two hydroxyl group in the aromatic ring, i.e., homo-HTy, lead to more efficient radical scavengers. The presence of a methoxy group in the meta- position relative to the carbon chain in the aromatic ring, *i.e.*, vanillyl alcohol and its derivatives does not significantly affect the radical scavenger properties in comparison to Ty and its esters. In the case of tyrosyl (**1a-d**) and vanillyl esters (**2a-d**), the newly synthetised lipophilic esters are less reactive than the corresponding alcohol. On the other hand, the series of *homo*-tyrosyl esters (**3a-d**) are more effective than the starting alcohol. Notably, the *homo*-HTy series has been used in the assay in a 10-fold lower concentration than the other derivatives demonstrating its higher antioxidant power. Finally, the obtained compounds have been submitted to antibacterial screening against a panel of Gram-negative and Gram-positive bacteria (Table 29, Figure 40). The mechanism of antimicrobial action of phenolic compounds and their esters is not well understood yet; they could affect membrane permeability or inhibit key enzymes that are involved in the growth and multiplication of microorganism cells [35].

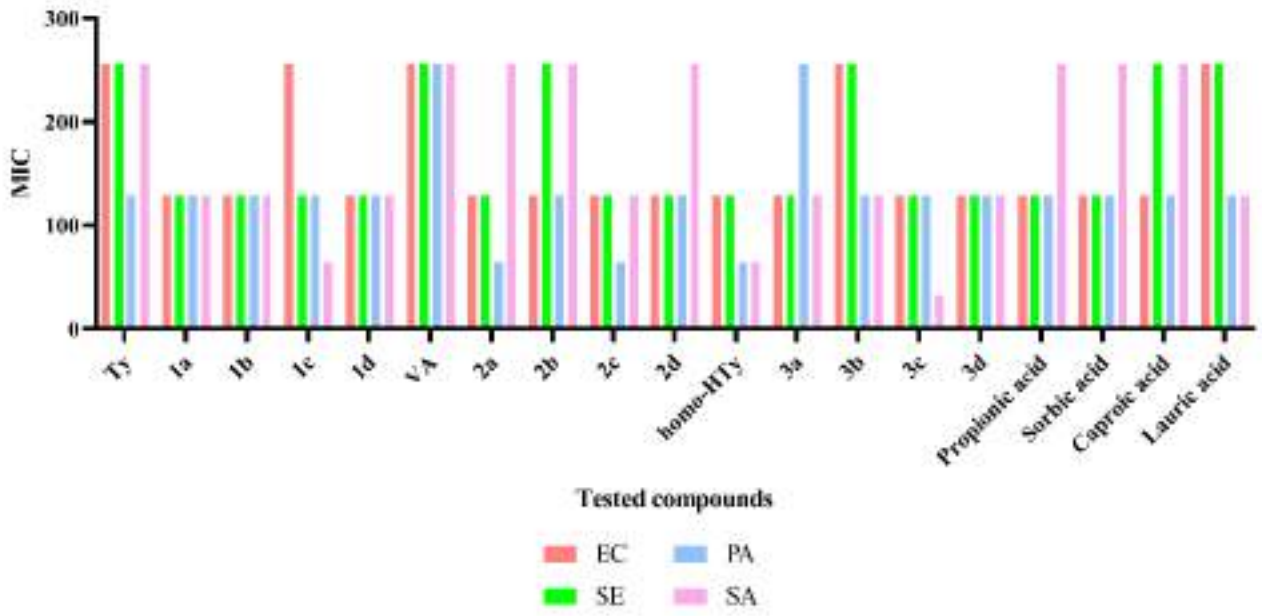


Figure 40. MIC of synthetised and parent compound against different bacteria.

Compound	*Ec* MIC (µg/mL)	*Se* MIC(µg/mL)	*Pa* MIC (µg/mL)	*Sa* MIC (µg/mL)
Ty	256	256	128	256
1a	128	128	128	128
1b	128	128	128	128
1c	256	128	128	64
1d	128	128	128	128
VA	256	256	256	256
2a	128	128	64	256
2b	128	256	128	256
2c	128	128	64	128
2d	128	128	128	256
homo-HTy	128	128	64	64
3a	128	128	256	128
3b	256	256	128	128
3c	128	128	128	32
3d	128	128	128	128
Propionic acid	128	128	128	256
Sorbic acid	128	128	128	256
Caproic acid	128	256	128	256
Lauric acid	256	256	128	128

Table 29 .Minimum inhibitory concentrations (MIC) (µg/mL) of tested compounds *Ec* = *E. coli*, *Se* = *S. enterica* subsp. *enterica* ser. Enteritidis, *Pa* = *P. aeruginosa*, *Sa* = *S. aureus*. All the tests were performed in triplicate.

In general, all the esters show a similar or slightly better antimicrobial activity against the selected bacteria in comparison with the parent alcohol and fatty acid. Interestingly, compound **3c** showed a MIC of 32 μM against the Gram-positive *Staphylococcus aureus*, 3-fold lower than against the Gram-negative bacteria selected for this study, being also more active than *homo*-HTy (MIC = 64 μM) and sorbic acid (MIC = 256 μM). Compound **1c** showed a MIC of 64 μM against *Staphylococcus aureus*, 3-fold lower than the corresponding starting alcohol Ty (MIC = 256 μM) and sorbic acid (MIC = 256 μM). Vanillyl ester **2a** and **2c** resulted more active on the Gram-negative *Pseudomonas aeruginosa* (MIC = 64 μM), being again 2- or 3-fold more active than the starting acids and vanillic alcohol. On the contrary, *E. coli* and *S. S. enterica* subsp. *enterica* ser. Enteritidis showed high MIC values versus all the tested molecules. Both cLogP and cLogS values reported in Table 30 indicate that the obtained esters should dissolve better in the lipid phase than in the aqueous phase, making possible an application in lipid-rich food matrices.

Compound	MW	cLogP	cLogS
Ty	138.17	1.147	-1.316
VA	154.16	0.647	-1.222
***homo*-HTy**	168.19	1.255	-1.290
1a	194.23	2.086	-1.996
2a	210.23	1.586	-1.902
3a	224.26	2.194	-1.970
1b	232.28	2.945	-2.350
1c	236.31	3.449	-2.806
2b	248.28	2.444	-2.256
2c	252.31	2.949	-2.712
3b	262.30	3.053	-2.324
3c	266.34	3.558	-2.780
1d	320.47	6.175	-4.426
2d	336.47	5.675	-4.332
3d	350.50	6.284	-4.400
1a	194.23	2.086	-1.996
2a	210.23	1.586	-1.902
3a	224.26	2.194	-1.970

Table 30. Calculated properties of tested compounds; Ty: tyrosol; VA: vanillic alcohol; homo-HTy: homo-hydroxytyrosol

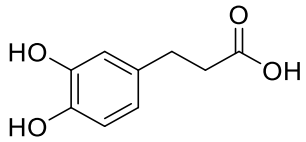
Derivatives characterised by a higher efficacy as radical scavengers belong to *homo*-HTy series (compounds **3a-d**), being compound **3b** the most effective (62% inhibition at 0.01 mM). Interestingly, compound **3c** also demonstrated the lowest MIC value against *Staphylococcus aureus.*

Materials and methods

Reagents and solvents were obtained from commercial suppliers and were used without further purification. NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer using the residual signal of the deuterated solvent as internal standard. ^{1}H chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) in hertz (Hz). Continuous flow biotransformations were performed using a R2+/R4 Vapourtec flow reactor or using Asia Flow Chemistry Syringe pumps (Syrris) equipped with an Omnifit® glass column (6.6 mm i.d. × 100 mm length). The temperature sensor sits on the wall of the reactors. Pressure was controlled by using back-pressure regulators. Hydrogenation reactions were recorded using an H-cube Mini Plus reactor (ThalesNano). HPLC analyses were carried out on a Jasco PU-980 pump equipped with a Jasco UV- 975 UV/Vis Detector. Column Luna 5u Silica (2) 100 Å, 250x4.6 mm; flow rate 1 mL/min; λ= 254 nm; mobile phase hexane / iPrOH 8:2. Retention time: **1d**: 3.6 min, Ty: 5.8 min. HR-MS instrument: Q-ToF Synapt G2-Si (Waters, Milford, MA, USA); source type: Electrospray ionization; data processing: MassLynx V4.2 software (Waters). Immobilised lipase B from *Candida antarctica* (Novozyme 435®) was purchased from Merck. 4-(3-Hydroxypropyl)benzene-1,2-diol (*homo*-HTy) was prepared from caffeic acid following a literature procedure [36].

Synthesis of 4-(3-hydroxypropyl)benzene-1,2-diol (*homo*-HTy)

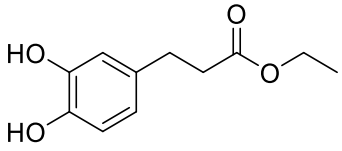
Synthesis of 3-(3,4-dihydroxyphenyl)propanoic acid



3-(3,4-dihydroxyphenyl)propanoic acid

A solution of caffeic acid in methanol was prepared (310 mg in 30 mL) and was submitted to hydrogenation reaction in a H-Cube Mini at 0.5 mL min^{-1}, T = 35 °C and P = 10 bar, Pd/C 5%. The solvent was evaporated to obtain the desired products in quantitative yields. White solid; yield 99.7 %; Rf = 0.5 (dichloromethane/ methanol 9:1 + 3 drops of acetic acid); ^{1}H NMR (300 MHz, MeOH-d_4) δ 6.70 – 6.57 (m, 2H), 6.52 (dd, *J* = 8.0, 2.2 Hz, 1H), 2.75 (t, *J* = 7.4 Hz, 2H), 2.51 (t, *J* = 7.3 Hz, 2H).

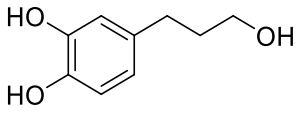
Synthesis of ethyl 3-(3,4-dihydroxyphenyl)propanoate



ethyl 3-(3,4-dihydroxyphenyl)propanoate

A solution of 3-(3,4-dihydroxyphenyl)propanoic acid (300 mg) in ethanol (10 mL) was cooled to 0 °C and magnetically stirred. $SOCl_2$ was added drop by drop to the solution and the mixture was kept under magnetic stirring for 5 min. The temperature was raised to room temperature and the mixture was left under magnetic stirring for 5 h. The reaction was monitored through TLC (dichloromethane/ methanol 9:1). The solvent was removed under pressure, the crude was dissolved in 20 mL of ethyl acetate and washed with $NaHCO_3$ 5% w/v (2 x 20 mL). The organic phase was dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure. Amber oil; yield = 94.4%; Rf = 0.77 (dichloromethane/ methanol 9:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.76 (d, *J* = 8.0 Hz, 1H), 6.71 (d, *J* = 2.1 Hz, 1H), 6.60 (dd, *J* = 8.1, 2.1 Hz, 1H), 5.92 (s, OH), 5.78 (s, OH), 4.13 (q, *J* = 7.1 Hz, 2H), 2.83 (t, *J* = 7.7 Hz, 2H), 2.57 (t, *J* = 7.8 Hz, 2H), 1.24 (t, *J* = 7.1 Hz, 3H).

Synthesis of 4-(3-hydroxypropyl)benzene-1,2-diol



4-(3-hydroxypropyl)benzene-1,2-diol

In a round bottom flask was added a solution 1M $LiAlH_4$ (7.3 mL) in 12 mL of dry THF under nitrogen and a solution of di BnCl (7.3 mmol, 1.5 eq) in 5 mL of dry THF. 4.9 mmol (1 eq.) of 3-(3,4-dihydroxyphenyl)propanoate were dissolved in 15 mL of dry THF and added dropwise to the stirred solution in the round bottom flask. The mixture was left under magnetic stirring for 2 h. 5 mL of wet ethyl acetate and 10 mL of water were added dropwise to quench the unreacted $LiAlH_4$. 2 N HCl was added to the mixture until the pH was around 1 and the water phase was extracted with ethyl acetate (6 x 10 mL). After the removal of the solvent, the crude was purified by column chromatography. Brown solid; yield = 79%; rf = 0.74 (dichloromethane/ methanol 8:2); ^{1}H NMR (300 MHz, MeOH-d_4) δ 6.65 (d, *J* = 8.0 Hz, 1H), 6.62 (d, *J* = 2.1 Hz, 1H), 6.49 (dd, *J* = 8.0, 2.1 Hz, 1H), 3.54 (t, *J* = 6.6 Hz, 3H), 2.57 – 2.45 (m, 2H), 1.84 – 1.68 (m, 2H).

General procedure for the flow esterification and in-line work-up

A glass column (i.d.: 6.6 mm) was packed with a previously prepared mixture of Novozyme 435® (692 mg) and powder molecular sieves 4 Å (692 mg) to obtain a packed bed reactor (PBR) with a final volume of 3.0 mL. A 0.1 M solution of the phenolic alcohol in CPME (20 mL) and a 0.3 M solution of the fatty acid in CPME (20 mL) were mixed in a T-piece and the resulting flow stream was directed into the reactor column kept at 60 °C. The total flow rate was 0.2 mL min^{-1} (residence time: 15 min). The exiting flow stream was flowed through a column (i.d.: 10 mm) packed with Amberlite IRA67 (2.8 g, total exchange capacity ≥ 1.60 eq/L FB form, PBR volume: 5.5 mL) at a total flow rate of 0.4 mL/min thanks to an inlet of CPME delivered through a third peristaltic pump. The organic solvent was collected and evaporated under pressure and, if necessary, the crude was purified by flash chromatography.

General procedure for the flow esterification with sorbic acid

A glass column (i.d.: 6.6 mm) was packed with a previously prepared mixture of Novozyme 435® (692 mg) and powder molecular sieves 4 Å (692 mg) (PBR volume: 3.0 mL). A 0.1 M solution of the phenolic alcohol in CPME (20 mL) and a 0.3 M solution of the sorbic acid in CPME (20 mL) were mixed in a T-piece and the resulting flow stream was directed into the reactor column kept at 60 °C. The total flow rate was 0.04 mL min^{-1} (residence time: 75 min). The organic solvent was collected and evaporated under pressure and the crude was purified by flash chromatography.

Compound characterisation

*4-Hydroxyphenethyl propionate (**1a**)* [37]*:* amber oil; yield: 80%; Rf = 0.56 (cyclohexane/EtOAc 7:3); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.08 (d, *J* = 8.7 Hz, 2H), 6.77 (d, *J* = 8.7 Hz, 2H), 4.24 (t, *J* = 7.1 Hz, 2H), 2.86 (t, *J* = 7.1 Hz, 2H), 2.31 (q, *J* = 7.6 Hz, 2H), 1.12 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.1, 154.5, 130.0, 129.6, 115.3, 65.2, 34.2, 27.6, 9.0.

*4-Hydroxyphenethylexa-2,4-dienoate (**1b**):* white solid; yield: 24%; Rf = 0.84 (cyclohexane/EtOAc 1:1); m.p.: 73.3 – 74.4 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31 – 7.16 (m, 1H), 7.09 (d, *J* = 8.1 Hz, 2H), 6.77 (d, *J* = 8.1 Hz, 2H), 6.27 – 6.04 (m, 2H), 5.76 (d, *J* = 15.4 Hz, 1H), 4.30 (t, *J* = 7.1 Hz, 2H), 2.89 (t, *J* = 7.1 Hz, 2H), 1.85 (d, *J* = 5.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.7, 154.5, 145.5, 139.8, 130.0, 129.7, 129.6, 118.6, 115.4, 65.2, 34.2, 18.6; HR-MS calculated for $C_{14}H_{16}O_3Na$ $[M+Na]^+$: 255.0997; found: 255.0994.

*4-Hydroxyphenethyl hexanoate (**1c**)* [38]*:* colourless oil; yield: 68%; Rf = 0.72 (cyclohexane/EtOAc 6:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.08 (d, *J* = 8.6 Hz, 2H), 6.76 (d, *J* = 8.6 Hz, 2H), 4.24 (t, *J* = 7.1 Hz, 2H), 2.86 (t, *J* = 7.1 Hz, 2H), 2.28 (t, *J* = 7.5 Hz, 2H), 1.68 – 1.52 (m, 2H), 1.37 – 1.21 (m, 4H), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.1, 154.3, 130.0, 129.8, 115.3, 65.0, 34.3, 34.2, 31.2, 24.6, 22.2, 13.8.

*4-Hydroxyphenethyl dodecanoate (**1d**)* [39]*:* white solid; yield: 90%; Rf = 0.66 (cyclohexane/EtOAc 7:3); m.p.: 40.5 – 42.0 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.08 (d, *J* = 8.1 Hz, 2H), 6.76 (d, *J* = 8.1 Hz, 2H), 4.24 (t, *J* = 7.1 Hz, 2H), 2.86 (t, *J* = 7.1 Hz, 2H), 2.28 (t, *J* = 7.0 Hz, 2H), 1.57 (m, 2H), 1.37 – 1.21 (m, 16H), 0.88 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.3, 154.4, 130.0, 129.7, 115.3, 65.1, 34.3, 34.2, 31.9, 29.6, 29.4, 29.3, 29.2, 29.1, 24.9, 22.6, 14.1

*4-Hydroxy-3-methoxybenzyl propionate (**2a**)* [40]*:* pale yellow oil; yield: 75%; Rf = 0.61 (cyclohexane/EtOAc 6:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.95 – 6.83 (m, 3H), 5.03 (s, 2H), 3.90 (s, 3H), 2.36 (q, *J* =7.6 Hz, 2H), 1.15 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.4, 146.4, 145.7, 127.9, 121.9, 114.3, 111.2, 66.3, 55.9, 27.6, 9.0.

4-*Hydroxy-3-methoxybenzylhexa-2,4-dienoate (**2b**):* white solid; yield: 30%; Rf = 0.58 (cyclohexane/EtOAc 6:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35 – 7.20 (m, 1H), 6.91 - 6.88 (m, 3H), 6.24 – 6.06 (m, 2H), 5.80 (d, *J* = 15.4 Hz, 1H), 5.64 (s, OH), 5.10 (s, 2H), 3.90 (s, 3H), 1.85 (d, *J* = 5.3 Hz, 3H). HR-MS calculated for $C_{14}H_{16}O_4Na$ $[M+Na]^+$: 271.0946; found: 271.0945.

*4-Hydroxy-3-methoxybenzyl hexanoate (**2c**)* [41]*:* colourless oil; yield: 67%; Rf = 0.74 (cyclohexane/EtOAc 6:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.95 – 6.83 (m, 3H), 5.64 (s, OH), 5.03 (s, 2H), 3.90 (s, 3H), 2.39 – 2.27 (m, 2H), 1.72 – 1.54 (m, 2H), 1.36 – 1.21 (m, 4H), 0.88 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.8, 146.4, 145.7, 128.0, 121.9, 114.3, 111.2, 66.2, 55.9, 34.3, 31.2, 24.6, 22.2, 13.8.

*4-Hydroxy-3-methoxybenzyl dodecanoate (**2d**)* [42]*:* yellow solid; yield: 61%; Rf = 0.74 (cyclohexane/EtOAc 6:4); m.p.: 35.5 – 36.9 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.94 – 6.83 (m, 3H), 5.64 (s, OH), 5.03 (s, 2H), 3.90 (s, 3H), 2.33 (t, *J* = 7.5 Hz, 2H), 1.61 (m, 2H), 1.30 - 1.25 (m, 16H), 0.93 – 0.82 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.8, 146.4, 145.7, 128.0, 121.9, 114.3, 111.2, 66.2, 55.9, 34.3, 31.8, 29.5, 29.4, 29.3, 29.2, 29.1, 24.9, 22.6, 14.1.

*3-(3,4-Dihydroxyphenyl)propyl propionate (**3a**):* light brown solid; yield: 76%, Rf = 0.55 (cyclohexane/EtOAc 1:1); m.p.: 65.6 – 67.9 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.77 (d, *J* = 8.1 Hz, 1H), 6.70 (d, *J* = 1.5 Hz, 1H), 6.60 (dd, *J* = 8.1, 1.5 Hz, 1H), 4.08 (t, *J* = 6.5 Hz, 2H), 2.57 (t, *J* = 6.5, 2H), 2.35 (q, *J* = 7.6 Hz, 2H), 1.90 (p, *J* = 6.5 Hz, 2H), 1.15 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.3, 143.6, 141.8, 134.0, 120.6, 115.4, 115.3, 63.8, 31.3, 30.1, 27.6, 9.1. HR-MS calculated for $C_{12}H_{15}O_4$ [M-H]$^-$: 223.0970; found: 223.0967.

*3-(3,4-Dihydroxyphenyl)propyl-hexa-2,4-dienoate (**3b**):* pale yellow solid; yield: 30%; Rf = 0.48 (cyclohexane/EtOAc 6:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 – 7.17 (m, 1H), 6.77 (d, *J* = 8.1 Hz, 1H), 6.70 (d, *J* = 2.0 Hz, 1H), 6.59 (dd, *J* = 8.1, 2.0 Hz, 1H), 6.27 – 6.05 (m, 2H), 5.92 (s, OH), 5.78 (d, *J* = 15.4 Hz, 1H), 5.77 (s, OH), 4.14 (t, *J* = 7.0 Hz, 2H), 2.58 (t, *J* = 7.0 Hz, 2H), 1.93 (p, *J* = 7.0 Hz, 2H), 1.86 (d, *J* = 5.3 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$**)** δ 167.9, 145.4, 143.6, 141.8, 139.8, 134.1, 129.7, 120.6, 118.6, 115.4, 115.2, 63.7, 31.3, 30.2, 18.6. HR-MS calculated for $C_{15}H_{17}O_4$ [M-H]$^-$: 261.1127; found: 261.1127.

*3-(3,4-Dihydroxyphenyl)propyl hexanoate (**3c**)* [43]*:* white solid; yield: 68%; Rf = 0.64 (cyclohexane/EtOAc 1:1); m.p.: 62.4 – 64.6 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.77 (d, *J* = 8.1 Hz, 1H), 6.70 (d, *J* = 1.4 Hz, 1H), 6.60 (dd, *J* = 8.1, 1.4 Hz, 1H), 5.44 (s, OH), 5.31 (s, OH), 4.07 (t, *J* = 6.6 Hz, 1H), 2.57 (t, *J* = 7.6 Hz, 3H), 2.31 (t, *J* = 7.6 Hz, 2H), 1.90 (p, *J* = 6.6 Hz, 2H), 1.63 (p, *J* = 7.6 Hz, 2H), 1.46 – 1.21 (m, 4H), 0.90 (t, *J* = 6.5 Hz, 3H). ^{13}C NMR (75 MHz**,** $CDCl_3$**)** δ 174.8, 143.6, 141.8, 134.0, 120.6, 115.4, 115.3, 63.8, 34.4, 31.3, 31.2, 30.2, 24.6, 22.2, 13.8.

*3-(3,4-Dihydroxyphenyl)propyl dodecanoate (**3d**)* [43]*:* white solid; yield: 59%; Rf = 0.69 (cyclohexane/EtOAc 1:1); m.p.: 65 – 66.9 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.77 (d, *J* = 8.0 Hz, 1H), 6.70 (d, *J* = 2.1 Hz, 1H), 6.60 (dd, *J* = 8.0, 2.1 Hz, 1H), 5.27 (s, OH), 5.12 (s, OH), 4.07 (t, *J* = 6.6 Hz, 2H), 2.57 (t, *J* = 7.5 Hz, 2H), 2.30 (t, *J* = 7.5 Hz, 2H), 1.90 (p, *J* = 6.6 Hz, 2H), 1.62 (p, *J* = 7.5 Hz, 2H), 1.37 – 1.21 (m, 16H), 0.88 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.6, 143.6, 141.7, 134.0, 120.6, 115.4, 115.2, 63.7, 34.4, 31.9, 31.3, 30.2, 29.5, 29.4, 29.3, 29.2, 29.1, 25.0, 22.6, 14.1.

DPPH radical-scavenging assay

Measurement of 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical-scavenging activity was performed using a commercial kit (Bioquochem, Asturie, Spain) following manufacturer's instructions. Briefly, samples were appropriately diluted in dimethyl sulfoxide (DMSO) and mixed with the DPPH solution provided by the kit. Trolox at different concentration was used to build the standard curve. Antioxidant Activity was determined by measuring absorbance at 517 nm by spectrophotometer (Eppendorf, Milan, Italy) and calculating the corresponding percentage of inhibition as reported in the kit instructions. All the assays were performed in triplicate and standard deviations varied between 1 and 5%.

Bacterial Strains and Culture Conditions

The evaluation was performed using *Escherichia coli* ATCC 25922 (*Ec*), *Salmonella enterica* subsp. *enterica* ser. Enteritidis ISM 8324 (*Se*), *Pseudomonas aeruginosa* IMV 1 (*Pa*) and *Staphylococcus aureus* ATCC 6538 (*Sa*). Bacteria were plated by streaking on blood agar plates (Tryptic Soy Agar + 5% sheep blood [Microbiol, Italy]) and incubated at 37 °C for 24 h under aerobic atmosphere. The Minimum Inhibitory Concentration (MIC) was determined using the microdilution assay, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute (CLSI) (2018) Performance Standards for Antimicrobial Susceptibility Testing. CLSI Approved Standard M100-S15. Clinical and Laboratory Standards Institute, Wayne). Briefly, all the strains were grown on Tryptic Soy Broth (TSB, Oxoid, Milan, Italy) and 3 or 4 isolated colonies were suspended in fresh sterile saline solution (9 g L^{-1} NaCl) to reach an initial concentration of 1.5 x 10^8 CFU/mL (equivalent to 0.5 MacFarland standard). One hundred microliters of the 1:100 diluted cell suspensions were dispensed into each well of a 96-well microtiter plate. The strains were exposed to 2-fold dilution series of each derivative (dissolved in DMSO). After incubation for 24 h at 37 °C under aerobic condition, the MICs were determined as the lowest dilution of molecules able to inhibit visible bacterial growth. All the tests were performed in triplicate.

Calculation of Selected Properties of Tested Compounds

Selected properties of the compounds (*i.e.*, molecular weights, cLogP, cLogS,) were calculated with OSIRIS DataWarrior. Calculated properties are summarized in Table 30.

Conclusions

The functional properties of phenolic compounds are not only influenced by the structural features of the phenolic moieties (*e.g.*, number, substitution pattern of OH groups), but also by the physico-chemical properties of the entire molecule (*e.g.*, hydrophilic/lipophilic balance, shape, polarizability). Hence, the same molecule can be efficient in one system (*e.g.*, emulsion) but not in another one (*e.g.*, bulk oil). LipophiliSation of phenolic derivatives with fatty acids represents an efficient strategy to obtain amphiphilic compounds that can be used as multifunctional additives in the food, cosmetic, and pharmaceutical industries, as they should conserve their other functional properties (UV A and UV B filters, antimicrobial, antiviral, bacteriostatic, etc.). The lipophiliSation should preserve the phenolic groups that are responsible for the antioxidant activity. In this context, a biocatalysed continuous flow approach for the synthesis of a series of phenolic derivatives selectively coupling the primary alcohol of a phenolic derivative with selected fatty acids was developed. The biotransformations were carried out in CPME as the solvent and the biocatalyst (Novozyme 435®) showed very good stability in this unconventional medium. The compounds were isolated in moderate to good yields and were tested as antimicrobials and radical scavengers. Esterification increases the lipophilicity of the starting phenolic compounds without losing their ability to neutralize free radicals and their antimicrobial properties, increasing their utility as preservative agents in lipid-rich media and their possible use in the food industry.

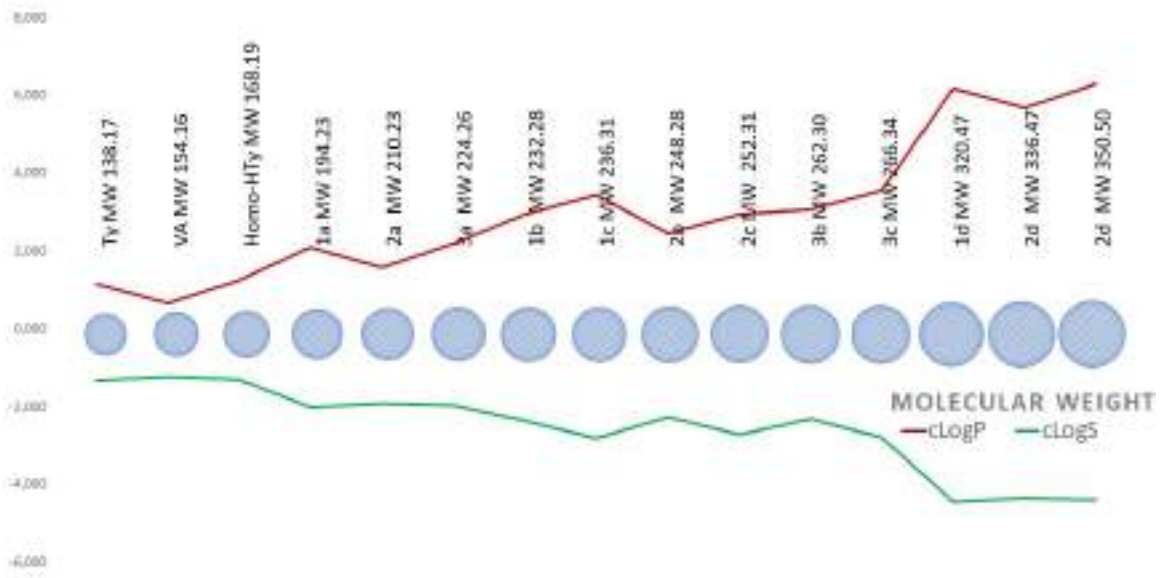


Figure 41. Schematic representation of calculated properties of tested compounds.

ii) Lipase mediated continuous synthesis of nature-inspired phenolic carbonate and carbamate derivatives as antimicrobial and antioxidant compounds

Introduction

The previous project, the synthesis of amphiphilic esters from natural phenols combined with fatty acids, resulted to be interesting both from a synthetic and biological point of view. The biocatalysed approach allowed to consider the final compounds as natural facilitating their use in nutraceutical and food applications. Biological assays for antimicrobial and antioxidant activities confirmed the preservation or enhancement of these biological activities while the lipophilicity was increased improving the bioavailability of the final compounds. A variety of active principles or secondary metabolites, *i.e.*, alkaloids, tannins, terpenoids essential oils, flavonoids, lectins, proteins, and polypeptides, quinones, coumarins, polyphenols and phenolic compounds, impart antimicrobial activities to many plant and animal products [44]. Moreover, according to literature, several alkyl-carbonate derivatives of hydroxytyrosol on the primary hydroxy group have been synthetised, showing that its antioxidant activity could be increased compared to that of the parent compound, heightening the molecule lipophilicity and dimensions [45].

In this context, it was interesting to broad the scope of the previous project developing a novel biocatalytic protocol for the synthesis also of carbonate and carbamate derivatives (compounds **1**-**5**) (Figure 42) of natural phenolic compounds as tyrosol (Ty) and hydroxytyrosol (HTy), which outstanding biological activities have been already covered in detail in chapter 2 [46]. Firstly, tyrosol and hydroxytyrosol carbonate derivatives have been designed and synthesised exploiting CaLB as biocatalytic agent in an unconventional organic media as dimethyl carbonate (DMC). Secondly, according to the prodrug strategy and Lipinski's rules, aiming at improving active molecules pharmacokinetic characteristics, especially focusing the attention on enhancing substrate lipophilicity (measured by LogP parameter), carbamate derivatives synthesis has been investigated. A similar sustainable protocol has been designed, exploiting *tert*-amyl alcohol as unconventional organic reaction solvent [47] in which CaLB could perform its aminolysis of carbonates without proceeding beyond the carbamate stage, exploiting amines with antioxidant properties, *i.e.*, tyramine and 1-(3-aminopropyl)imidazole [48]. It is noteworthy the novelty of this second phosgene-free procedure, exploiting a green biocatalysed continuous synthesis of a key structural pharmaceutical motif, important both for its chemical and proteolytic stability, and its capability to permeate cell membranes. The antimicrobial activities and their ability to scavenge radicals of the newly synthetised compounds had been tested.

Results and discussion

For the synthesis of this small series of carbonate (compounds 1-3) and carbamate derivatives (compounds 4 and 5) of tyrosol and hydroxytyrosol (Figure 42), a batch scouting activity was carried out, focusing the attention on greener and safer protocols that could ensure the synthesis of the desired products. Secondly, after the reaction conditions were optimised, the processes were performed under continuous flow conditions. Lipase from *Candida antarctica* B was employed as the only versatile biocatalyst for the production of the different compounds.

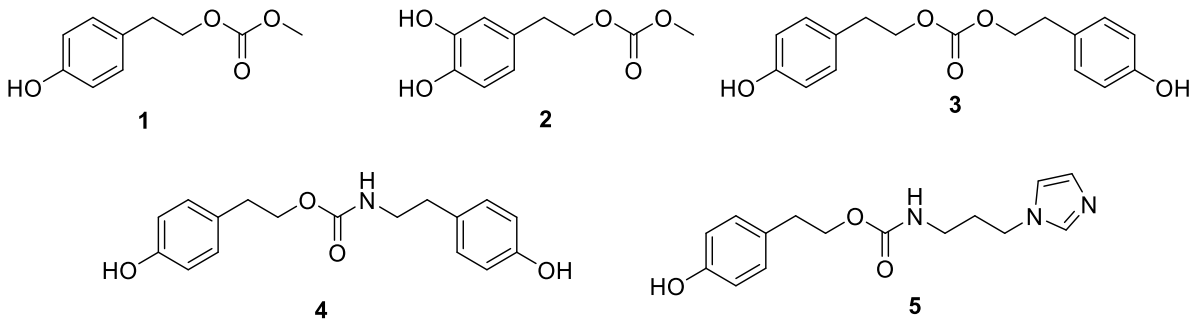
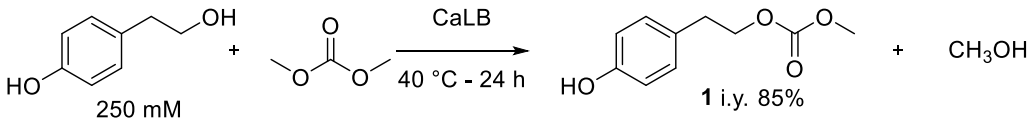


Figure 42. Chemical structure of the synthetised compounds.

Carbonate synthesis

The synthesis of methyl carbonate derivative of tyrosol (**1**) using immobilised CaLB as biocatalyst was studied as a model reaction and the optimised procedure was then applied to the synthesis of compound **2** while compound **1** itself was used a substrate for the synthesis of compound **3**. Tyrosol (100 mM) was dissolved in dimethyl carbonate (DMC), employed both as reagent and reaction medium to enhance product formation. DMC is considered, according to the solvent selection guide, greener alternative to common chemicals such as methyl ethyl ketone, ethyl acetate, methyl isobutyl ketone, and most of the other ketones [30]. It is produced from CO_2 and possesses properties of non-toxicity and biodegradability which makes it a true green reagent to use in syntheses [49]. The enzyme in its immobilised form was added and after 6 h at 40 °C, the reaction was complete. With the aim in mind of increasing the productivity, higher concentration of tyrosol were tested and the highest concentration achievable without observing precipitation was 250 mM. However, in this experimental condition the reaction was not complete after 24 h, even raising the temperature to 60 °C. Nevertheless, the isolated yield resulted to be 85 %, despite the fact that the reaction was not complete.



Scheme 32. Batch synthesis to obtain methyl carbonate derivative of tyrosol.

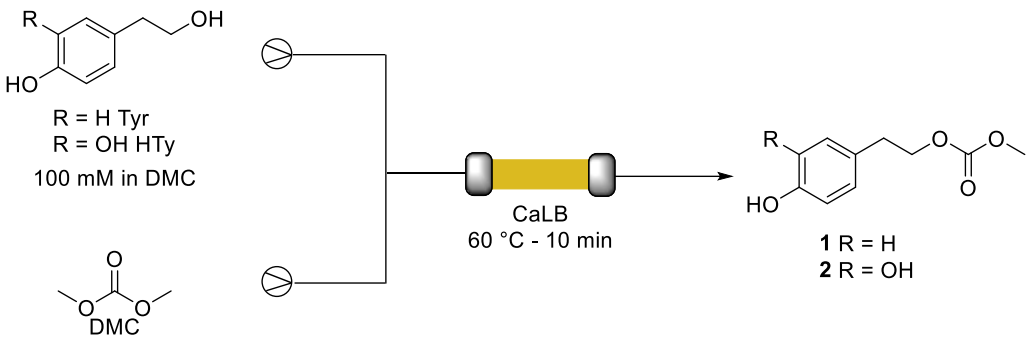
Moving to a continuous approach different set-up and experimental conditions were considered, evaluating several temperatures (*i.e.*, 40° C, 50° C and 60° C), tyrosol concentrations (*i.e.*, 100 mM, 200 mM and 250 mM), and residence times (*i.e.*, 5, 10 and 15 min).

Entry	Tyrosol concentration	Temperature	Residence time	Complete conversion
1	100 mM	50 °C	15 min	Y
2	100 mM	40 °C	15 min	Y
3	100 mM	40 °C	10 min	N
4	100 mM	40 °C	5 min	N
5	100 mM	60 °C	10 min	Y
6	250 mM	60 °C	10 min	N
7	200 mM	60 °C	15 min	N

Table 31. Different reaction parameters evaluated for the optimisation of the continuous synthesis of compound **1**.

From the results obtained, reported in Table 31, it was observed that the reaction in the continuous system (Scheme 33) was complete after only 15 min both at 50 °C or lowering the temperature at 40 °C (entries **1** and **2**). Decreasing the residence time maintaining the same temperature resulted in a partial conversion of tyrosol into product **1** (entries **3** and **4**) but raising the temperature at 60 °C in only 10 min complete conversion was obtained (entry **5**). To improve the productivity of the system higher concentration of tyrosol were tested (entries **6** and **7**), however the biotransformation was not complete. The switch from a batch traditional set up to a continuous allowed to reduce the reaction time, simplify the work-up and increase the yield: the former needed 6 h to bring the reaction to completeness, filtration under pressure and flash chromatography purification steps were necessary to isolate the pure product (**1**), and the best yield achieved was 93 %; the latter needed only 10 min of residence time to bring the reaction to completeness, did not require downstream processes giving a quantitative yield. The optimised conditions were then applied to the synthesis of compound **2**. Considering the hydroxytyrosol low availability in nature and its considerable price, I employed the continuous procedure described in chapter 2 for the continuous biocatalysed oxidation

of tyrosol into hydroxytyrosol [50]. Subsequently, the desired product was used as starting reagent for the synthesis of 3,4-dihydroxyphenetyl methyl carbonate (**2**) (Scheme 33).



Scheme 33. Optimised synthesis of compounds **1** and **2** in a flow system.

According to literature [51-52], the synthesis of compound **3** was difficult and far away from being environmentally friendly and sustainable. In fact, the first step of its synthesis is usually performed under inert atmosphere, adding triphosgene dropwise to the reactor vessel containing a tyrosol solution in anhydrous tetrahydrofuran. Secondly, always in an inert environment, tyrosol chloroformate was the substrate of tyrosol nucleophilic attack, obtaining *bis*(4-hydroxyphenethyl) carbonate (**3**) [51]. Considering the well-known triphosgene corrosivity, toxicity together with its environmental and toxicological issues, and the carbonylation reactions drawbacks as the toxicity of CO and risks associated to potentially explosive mixture of CO and O_2, non-phosgene strategies and catalytic carbonylation reactions could be highly attractive. In recent years, the attention was focused on eco-compatible methodologies employing non-toxic and biodegradable chemicals, reducing waste generation and energy requirements. In addition, to overcome phosgenation step some halogen-free processes (*e.g.*, oxidative carbonylation of alcohols, alcoholysis of urea, transesterification of dimethyl carbonate with alcohols and synthesis of organic carbonate from CO_2) have been studied. Additionally, compound **3** has been recently described as monomer of a new class of biodegradable, aromatic polycarbonates deriving from tyrosol. In fact, *bis*(4-hydroxyphenethyl) carbonate (**3**) has been used as one of the starting structures for polymerization steps, achieving alternating or scrambled polycarbonates sequences [52]. A one-pot approach starting directly from tyrosol was tested, reason why a batch study was performed. *Tert*-Amyl alcohol is an intermediate polar solvent (logP 1.5) that has been demonstrated to keep a high catalytic activity of lipase because of its large steric hindrance effect [53]. Combined with its safety profile, low freezing point (in comparison to *t*-BuOH), and ability to solubilize polar compounds, it was chosen as unconventional medium for the biocatalysed synthesis of the following compounds

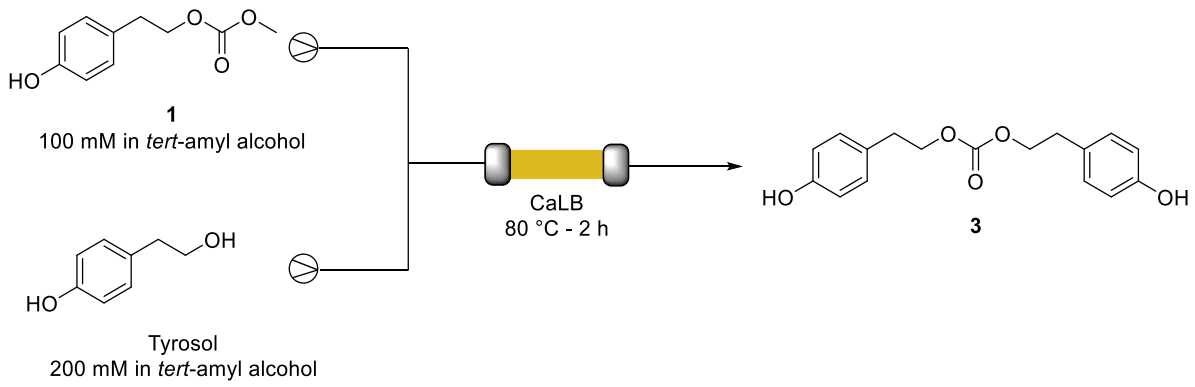
[54]. A solution of tyrosol, in *tert*-amyl alcohol, DMC and immobilised CaLB was heated at 60 °C. After 48 h the product **3** and compound **1** were detected in the mixture, but the reaction was too slow to be considered useful, reason why this synthetic approach has been dismissed. Indeed compound **1** was used as a starting point for the synthesis of compound **3**. To a solution of compound **1** in *tert*-amyl alcohol (100 mM), tyrosol (200 mM) and immobilised CaLB were added. The reaction has been monitored by TLC analysis for 4 days. The crude has been purified and the product was isolated in 23% yield.

The following step was moving into a continuous system for the biotransformation of compound **1** into *bis*(4-hydroxyphenethyl) carbonate **3**, with the aim of developing a productive, efficient, and automated process. Different experiments were performed changing residence times (30, 60, 120 and 180 min) at 80° C (Table 32). This time to identify the best set up the conversion was checked by HPLC method.

Entry	Residence time (min)	Conversion a %
1	30	21
2	60	42
3	120	55
4	180	60

Table 32. Optimisation of the continuous synthesis of compound **3**. [a] HPLC conversion.

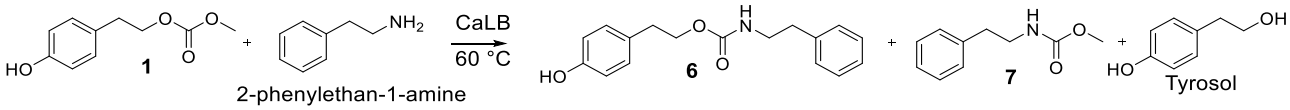
Since the difference in the conversion between 120 min and 180 min was not significant, the final set-up was settled for a residence time of 2 h which allowed to obtain a conversion of 55% (Scheme 34). The switch from a batch traditional set up to a continuous one, improved the reaction time (2 h vs 4 day) and reduced the work-up steps, maintaining the same isolated yield of 23%.



Scheme 34. Continuous synthesis of compound **3**.

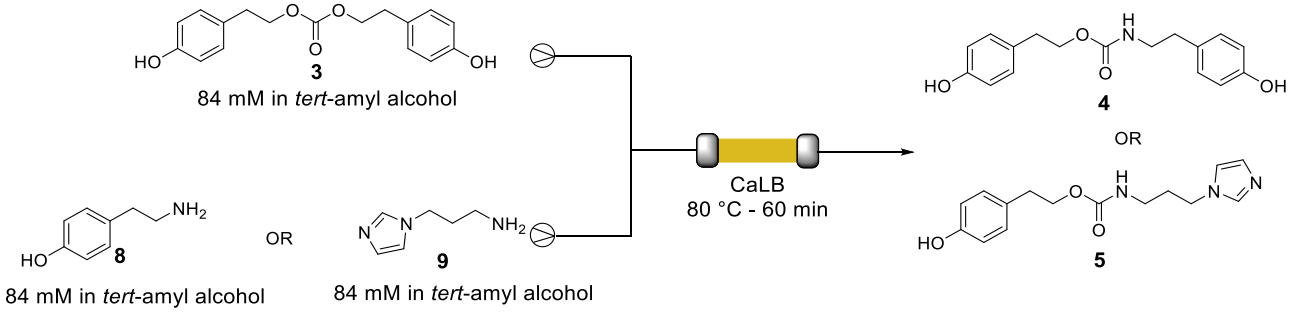
Carbamate synthesis

Once compound **3** was efficiently synthetised in a continuous set-up it was employed as a starting point to develop an efficient methodology for the biocatalysed synthesis of carbamate derivatives instead of compound **1**. In this way, tyrosol was the by-product and not MeOH, which even if is considered an optimal leaving group for the nucleophilic attack reaction, in presence of lipase it was demonstrated to rapidly take part in the transesterification of carbamates bringing to the formation of by-product **7**.



Scheme 35. Formation of by-product **7** using compound **1** as starting compound.

In addition, the tyrosol produced could be easily purified and reused, supporting the circular economic model. As a model reaction, the nucleophile 2-phenylethylamine was used for the batch studies and for continuous set-up optimisation. The reaction took place once again in *tert*-amyl alcohol at 60 °C for 28 h and after purification compound **6** was isolated (31% yield). With the aim of developing a more efficient and automated process, the process was moved to continuous approach. Then, I move to the continuous synthesis (Scheme 36) of compound **4** and **5**, using tyramine (**8**) and 1-(3-aminopropyl)imidazole (**9**) as suitable nucleophiles. After only 1hour of residence time at 80 °C the products were isolated in 26% (compound **4**) and 20% (compound **5**) yields.



Scheme 36. Continuous biocatalysed synthesis of compound **4** and **5**.

Biological assay and results

All the synthetised compounds, with the exception, at the moment, of compound **2**, were tested as antimicrobial agents and as antioxidant agents. Firstly, they were tested by Dr Piera Anna Martino

(Department of Biomedical, Surgical and Dental Sciences) as antimicrobial agents against both Gram-negative and Gram-negative bacteria, *i.e.*, *Escherichia coli, Salmonella enterica Enteritidis, Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Compound	EC MIC (μg/mL)	SE MIC (μg/mL)	PA MIC (μg/mL)	SA MIC (μg/mL)
Tyrosol	128	128	128	128
1	128	128	128	128
3	128	128	128	128
4	128	128	128	128
5	128	128	128	128

Table 33. MIC of compounds **1**, **3**, **4** and **5**. Legend: EC = *Escherichia coli*, SE = *Salmonella enterica Enteritidis*, PA = *Pseudomonas aeruginosa*, SA = *Staphylococcus aureus*

According to the results reported in Table 33, the antimicrobial activity of tyrosol derivatives remained unaltered even after its synthetic derivatization as carbonate or carbamate. Thus, the compound lipophilicity has been improved, leaving unchanged the tyrosol antimicrobial properties. Then their efficiency as radical scavengers was evaluated performing a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay [34].

Compounds	(%) Inhibition
Tyrosol (0.1 mM)	45
1 (0.1 mM)	46
3 (0.1 mM)	54
4 (0.1 mM)	55
5 (0.1 mM)	43

Table 34. Determination of the free radical scavenging capacity of compounds **1**,**3**,**4** and **5**.

According to the results (Table 34), the antiradical activity of compounds **1**, **3**, **4** and **5** were similar to the one of the parent compound tyrosol; a slight increase of the inhibition percentage was observed when a second phenolic group was inserted in the chemical structure, *i.e.*, compounds **3** and **4**. As a result, increasing the lipophilicity of the natural molecule had no detrimental effect on the radical scavenger activity. Moreover, selected properties of the compounds (*i.e.*, molecular weights, cLogP, cLogS, H-Acceptors, H-Donors, drug likeness and total surface area) were calculated with OSIRIS Data Warrior.

Compound	Total MW	cLogS	cLogP	H-Acceptors	H-Donors	Drug likeness	Total Surface Area
Ty	138.165	-1.316	1.1467	2	2	-4.1391	113.48
HTy	154.164	-1.02	0.801	3	3	-4.1391	119.83
1	196.201	-2.041	1.8345	4	1	-7.3607	157.14
2	302.325	-3.18	3.337	5	2	-3.6692	238.51
3	301.341	-3.044	2.8628	5	3	-13.174	239.97
4	289.334	-1.59	1.4524	6	2	-9.86	235.34
5	212.2	-1.745	1.4888	5	2	-7.3607	163.49

Table 35. Selected properties of the synthetized compound calculated with OSIRIS Data Warrior.

The calculated values reported in Table 35 show that the obtained carbonate and carbamate should dissolve better in the lipid phase than in the aqueous one, making possible an application in lipid-rich matrices for food, cosmetic and pharma applications.

Materials and methods

Reagents and solvents were obtained from commercial suppliers and were used without further purification. NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer using the residual signal of the deuterated solvent as internal standard. ^{1}H chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) in hertz (Hz). Continuous flow biotransformations were performed using a R2+/R4 Vapourtec flow reactor or using Asia Flow Chemistry Syringe pumps (Syrris) equipped with an Omnifit® glass column (i.d 6.6 mm.× 100 mm length and i.d. 10 mm × 100 mm length). The temperature sensor sits on the wall of the reactors. Immobilised lipase from *Candida antarctica* B (CaLB - Novozyme®) was purchased from Merk (Milan, Italy). Hydroxytyrosol was synthetised as previously reported (chapter 2). Analytical thin layer chromatography (TLC) was carried out on TLC plates precoated with silica gel 60 F_{254}, to monitor the reaction course through an UV light (254 nm). Spots were further evidenced by spraying different staining solutions: a dilute alkaline solution of $KMnO_4$, an acidic solution of vanillin in ethanol or an alcoholic solution of ninhydrin. HPLC analyses were performed using a Jasco PU-980 pump, exploiting ChromNAV software and using a LiChroCART® 125-4 LiCrosphere ®, Si60 (5μm) column. An UV detector at 254 nm was used for the detection of the carbonates and carbamates synthetized.

HPLC method

Jasco PU-980 Intelligent HPLC pumps equipped with Jasco UV-975 Intelligent UV/VIS Detector (λ = 254 nm), linked to a computer with an interface box (Jasco LC-Net II/ADC), were employed to perform HPLC analyses. LiChroCART ® 125-4 LiChrosphere ®, Si60 (5 μm), Merck Millipore, was the column used. For the analysis of compound **1** and **3** a gradient *n*-hexane/*iso*-propanol (95:5 from 0 to 10 min, 70:30 from 10 to 20 min, 95:5 from 20 to 30 min) was used. Retention times: **1 =** 5 min, **3 =** 10 min, tyrosol = 14 min. For the analysis of compound **6** a gradient *n*-hexane/*iso*-propanol (98:2 from 0 to 3 min, 95:5 from 3 to 14 min, 70:30 from 14 to 22 min, 10:90 from 22 to 28 min, 98:2 from 28 to 60 min). Retention times: **6** = 10.8 min, **2 =** 13 min, tyrosol = 18 min.

Batch synthesis of 4-hydroxyphenethyl methyl carbonate -1 (**1**)

To a solution of tyrosol (691 mg, 5.0 mmol), in dimethyl carbonate (DMC, 20 mL, 250 mM), 150 mg of *Candida antarctica* lipase type B, immobilised on polystyrene beads, was added into a round bottom flask. The reaction proceeded at 40 °C, under stirring for 20 h. The reaction course was monitored by thin layer chromatography (cyclohexane/ethyl acetate 6:4). The reaction mixture was filtered under vacuum. DMC was evaporated under reduced pressure; the crude was then purified

through flash chromatography (cyclohexane/ethyl acetate 7:3) to obtain the corresponding 4-hydroxyphenethyl methyl carbonate (**1**). Yield 85 %.

Continuous synthesis of 4-hydroxyphenethyl methyl carbonate -1 (1) and 3,4-dihydroxyphenetyl methyl carbonate (2)

A stock solution of tyrosol or hydroxytyrosol in DMC (4.2 mmol, 42 mL) was injected into a bioreactor packed with 545 mg of CaLB (volume: 1.6 mL) with a flow rate of 0.157 Ml min^{-1}. DMC was employed as flow stream. The reaction was performed at 65 °C with a residence time of 10 min. The resulting crude was collected, the solvent was evaporated under reduced pressure to give the desired product that was used without further purifications. (**1**) yield 99%. (**2**) yield 99%.

Batch synthesis of *bis*(4-hydroxyphenethyl) carbonate (3)

To a solution of compound **1** (220 mg, 1.12 mmol, 1 eq.) in *tert*-amyl alcohol (11.2 mL, 100mM), tyrosol (309 mg, 2.24 mmol, 2 eq.) and 200 mg of CaLB, immobilised on polystyrene beads, were added. The reaction proceeded at 60 °C, under stirring for 4 days. The reaction course was monitored by TLC (cyclohexane/ethyl acetate 6:4). The reaction mixture was filtered under vacuum and the solvent was evaporated under reduced pressure. The resulting crude was purified by column chromatography (cyclohexane/ethyl acetate 8:2). (**3**) yield: 23 %.

Continuous synthesis *bis*(4-hydroxyphenethyl) carbonate (3)

To a solution of compound **1** (584 mg, 2.9 mmol, 1 eq.) in *tert*-amyl-alcohol (30 mL, 100 mM), tyrosol was added (822 mg, 5.9 mmol, 200 mM, 2 eq.), obtaining a colourless clear solution. The flow stream was *tert*-amyl alcohol. The solution was injected into a bioreactor packed with 1.3 g CaLB, immobilised on polystyrene beads (volume: 3 mL), with a flow rate of 25 μL min^{-1}, the reaction was performed at 80 °C for a total residence time of 2 h. The resulting crude was collected and analysed by thin layer chromatography (cyclohexane/ethyl acetate 6:4). The solvent was evaporated under reduced pressure and the crude was purified by flash column chromatographic (cyclohexane/ ethyl acetate 8:2). (**3**) yield: 23 %.

Continuous synthesis of 4-hydroxyphenethyl (4-hydroxyphenethyl)carbamate (4) and 4-hydroxyphenethyl (3-(1*H*-imidazol-1-yl)propyl)carbamate (5)

To a solution of compound **3** (127 mg, 4.2 mmol) in *tert*-amyl alcohol (5 mL, 84 mM), the nucleophile (**8** or **9**, 4.2 mmol, 84 mM) was added and the solution directed into a bioreactor packed with 600 mg of CaLB (volume: 1.7 mL). The reaction was performed at 80 °C for a total residence

time of 1 hour (total flow rate: 28.8 μL min^{-1}). The exiting solution was collected in a flask and analysed by thin layer chromatography (cyclohexane/ethyl acetate 7:3, for compound **4** and dichloromethane/methanol 9:1 for compound **5**). The solvent was evaporated under reduced pressure and the crude was purified through flash column chromatography (cyclohexane/ethyl acetate 7:3, for compound **4**, whereas dichloromethane/methanol gradient from 95:5 to 8:2, for compound **5**). The solvent was evaporated under reduced pressure to give the desired compound **4** (yield 26%) or **5** (yield 20%).

Compounds characterisation

*4-hydroxyphenethyl methyl carbonate -1 (**1**)* [55]*:* white solid; Rf = 0.66 (cyclohexane/ethyl acetate 6:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.09 (d, *J* = 6.8 Hz, 2H), 6.77 (d, *J* = 6.8 Hz, 2H), 4.87 (s, 1H, OH), 4.30 (t, *J* = 7.15 Hz, 2H), 3.77 (s, 3H), 2.91 (t, *J* = 7.15 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.8, 154.2, 130.0, 129.2, 115.3, 68.6, 54.7, 34.2.

*3,4-dihydroxyphenetyl methyl carbonate (**2**)* [56]: colourless oil; Rf = 0.52 (cyclohexane/ethyl acetate 6:4); ^{1}H NMR (300 MHz, CD_3OD) δ 6.71-6.63 (m, 2H), 6.55-6.49 (m, 1H), 4.22 (t, *J* = 7.1 Hz, 2H), 2.78 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 155.9, 144.8, 143.5, 128.8, 119.7, 115.5, 114.9, 68.6, 53.7, 34.0.

*bis(4-hydroxyphenethyl) carbonate (**3**)* : pale yellow solid; Rf = 0.45 (cyclohexane/ethyl acetate 6:4); ^{1}H NMR (300 MHz, CD_3OD) δ 7.02 (d, *J* = 8.4, 4H), 6.70 (d, *J* = 8.4, 4H), 4.21 (t, *J* = 7.0, 4H), 2.82 (t, *J* = 7.0, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ 155.7, 155.2, 129.5, 128.1, 114.8, 68.3, 33.8.

*4-hydroxyphenethyl (4-hydroxyphenethyl)carbamate (**4**)*: yellow oil; Rf = 0.22 (cyclohexane/ethyl acetate 7:3); ^{1}H NMR (300 MHz, CD_3OD) δ 7.14 – 6.89 (m, 4H), 6.75 – 6.63 (m, 4H), 4.13 (t, *J* = 7.0, 2H), 3.23 (t, *J* = 7.4, 2H), 2.78 (t, *J* = 7.0, 2H), 2.65 (t, *J* = 7.4, 2H). ^{13}C (75 MHz, CD_3OD) δ 155.6, 155.4, 129.4, 129.3, 128.7, 114.7, 65.3, 42.2, 34.8, 34.3.

*4-hydroxyphenethyl (3-(1H-imidazol-1-yl)propyl)carbamate (**5**)*: colourless oil; Rf = 0.44 (dichloromethane/ methanol 9:1); ^{1}H NMR (300 MHz, CD_3OD) δ 7.64 (s, 1H), 7.11(s, 1H), 7.04 (d, *J* = 8.45 Hz, 2H), 6.95 (s, 1H), 6.70 (d, *J* = 8.45 Hz, 2H), 4.17 (t, *J* = 6.95 Hz, 2H), 4.01 (t, J_1 = 6.9 Hz, 2H), 3.06 (t, J_2 = 6.5 Hz, 2H), 2.80 (t, *J* = 6.95 Hz, 2H), 1.92 (p, J_1 = 6.9 Hz, J_2 = 6.5 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 157.7, 155.6, 137.0, 129.5, 128.7, 127.5, 119.1, 114.7, 65.3, 43.9, 37.1, 34.3, 30.9.

DPPH radical-scavenging assay

Measurement of 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical-scavenging activity was performed using a commercial kit (Bioquochem, Asturie, Spain) following manufacturer's instructions. Briefly, samples were appropriately diluted in dimethyl sulfoxide (DMSO) and mixed with the DPPH solution provided by the kit. Trolox at different concentration was used to build the standard curve. Antioxidant Activity was determined by measuring absorbance at 517 nm by spectrophotometer (Eppendorf, Milan, Italy) and calculating the corresponding percentage of inhibition as reported in the kit instructions. All the assays were performed in triplicate and standard deviations varied between 1 and 5%.

Bacterial Strains and Culture Conditions

The evaluation was performed using *Escherichia coli* ATCC 25922 (*Ec*), *Salmonella enterica* subsp. *enterica* ser. Enteritidis ISM 8324 (*Se*), *Pseudomonas aeruginosa* IMV 1 (*Pa*) and *Staphylococcus aureus* ATCC 6538 (*Sa*). Bacteria were plated by streaking on blood agar plates (Tryptic Soy Agar + 5% sheep blood [Microbiol, Italy]) and incubated at 37 °C for 24 h under aerobic atmosphere. The Minimum Inhibitory Concentration (MIC) was determined using the microdilution assay, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute (CLSI) (2018) Performance Standards for Antimicrobial Susceptibility Testing. CLSI Approved Standard M100-S15. Clinical and Laboratory Standards Institute, Wayne). Briefly, all the strains were grown on Tryptic Soy Broth (TSB, Oxoid, Milan, Italy) and 3 or 4 isolated colonies were suspended in fresh sterile saline solution (9 g/L NaCl) to reach an initial concentration of 1.5 x 10^8 CFU/mL (equivalent to 0.5 MacFarland standard). One hundred microliters of the 1:100 diluted cell suspensions were dispensed into each well of a 96-well microtiter plate. The strains were exposed to 2-fold dilution series of each derivative (dissolved in DMSO). After incubation for 24 h at 37 °C under aerobic condition, the MICs were determined as the lowest dilution of molecules able to inhibit visible bacterial growth. All the tests were performed in triplicate.

Calculation of Selected Properties of Tested Compounds

Selected properties of the compounds (*i.e.*, molecular weights, cLogP, cLogS,) were calculated with OSIRIS DataWarrior.

Conclusions

In the present work, the possibilities of lipase from *Candida antartica* B as a versatile synthetic tool for the production of biologically active carbonate and carbamate starting from phenolic compounds as tyrosol and hydroxytyrosol has been investigated. Thanks to the exploitation of the immobilised form of the biocatalyst in a packed bed reactor, reaction time, work-up efficiency and safety were increased compared to the traditional batch synthesis. In only 10 min, compound **1** has been synthetised under flow conditions with a specific reaction rate of 28.8 μmol $min^{-1}g^{-1}$, compared to the 6 h batch procedure which was able to produce a specific reaction rate 18.5 μmol $min^{-1}g^{-1}$, achieving a yield > 99%, without further downstream processes required [57]. With the same optimised protocol also compound **2**, the methyl carbonate derived of hydroxytyrosol was prepared. A biocatalysed phosgene-free synthesis of the biodegradable monomer compound **3**, used in this project also as reaction intermediate, has been optimised in continuous, dramatically reducing the reaction time from 4 days to 2 h of residence time. Moreover, compound **3** opened the way for the continuous biocatalysed synthesis of carbamate derivatives **4** and **5**. Immobilised CaLB has been recycled several times before its catalytic activity decreased, and all the trials have been performed at temperature between 40 - 80 °C, without compromising the enzyme activity, exploiting a non-conventional medium, *i.e.*, *tert*-amyl alcohol. According to the biological assays, the increased lipophilicity of the new compound did not affect their antimicrobial activities and ability to scavenge radicals improving at the same time their bioavailability. In the near future, this protocol will be tested towards new alcohols and amines, obtaining new carbonate and carbamate derivatives for further structure activity relationship studies.

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[57] Specific reaction rates in batch and continuous-flow systems were calculated using the following equations:

$$r_{batch} = \frac{\eta_p}{t \times m_B} \text{ (mol/min g)}$$

Where [n_p] is the amount of product (expressed as µmol), t is the reaction time (expressed as min), and m_B [g] is the amount of biocatalyst employed.

$$r_{flow} = \frac{[P] \times f}{m_B} \text{ (mol/min g)}$$

Where [P] is the product concentration flowing out of the reactor (expressed as µmol/mL), f is the flow rate (expressed as mL/min), and m_B [g] is the amount of biocatalyst loaded in the column. Comparison of the kinetics of the same reaction in a batch or flow-mode was made at similar degrees of conversion.

FIGURES

SCHEMES

TABLES