TÉCNICO LISBOA

Enantiomeric Resolution of a Non-racemic Mixture of Methyl 2-phenylpropanoate Using Marine Lipases

Miguel Alexandre Ricardo da Cunha

Thesis to obtain the Master of Science Degree in

Biological Engineering

Supervisors: Prof. Carla da Conceição Caramujo Rocha de Carvalho
Dr. Filipe Daniel Ramos de Carvalho

Examination Committee

Chairperson: Prof. Ana Margarida Nunes da Mata Pires de Azevedo
Supervisor: Prof. Carla da Conceição Caramujo Rocha de Carvalho
Member of the Committee: Prof. Pedro Carlos de Barros Fernandes

January 2021

Preface

The work presented in this thesis was performed at iBB - Institute for Bioengineering and Biosciences of Instituto Superior Técnico, Universidade de Lisboa, during the period of February-October 2020, under the supervision of Prof. Carla da Conceição Caramujo Rocha de Carvalho and Dr. Filipe Daniel Ramos de Carvalho. Due to the COVID-19 pandemic, laboratorial work was limited to the months of July, September and October 2020.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Acknowledgments

I would like to thank Prof. Carla Carvalho for all the support and sharing of knowledge, without whom this thesis would not be possible.

I would also like to thank Dr. Filipe Carvalho for the helpful discussions into the topic, PhD candidate Ricardo Pereira without whom any work in the laboratory would not be possible and PhD candidate Carlos Rodrigues for the support provided in the laboratory.

Abstract

The biological properties and applications of chiral compounds depend on their configuration. Biotransformation allows the enantioresolution of chiral compounds under mild conditions while allowing the increasingly important *natural* label.

Lip_1 was found to selectively hydrolyse *(S)*-methyl 2-phenylpropanoate (MPP). Under the appropriate enzyme and substrate concentrations, the hydrolysis rate was 425.6 μmol MPP/mg Lip_1.h with an enantiomeric excess (ee) of 53.7 %. Specific Lip_1 activity did not show significant change in the presence of up to 20 %(v/v) methanol or dimethyl sulfoxide (DMSO) in the reaction medium. At 30 %(v/v) of methanol or DMSO, Lip_1 activity was reduced 3.14-fold and increased 6% with ee of 6.88 % and 55.7 %, respectively. A four-fold increase in the reaction volume led to a 45 % decrease in Lip_1 activity with an ee of 28.2 %.

Lip_2 was found to selectively hydrolyse the *(R)*-MPP. Under the appropriate enzyme and substrate concentrations, the hydrolysis rate was 0.344 μmol MPP/mg Lip_2.h with an ee of -10.9 %. Adding methanol up to 20 %(v/v) to the reaction system led to an increase of 37 % of Lip_2 specific activity with an ee of 6.23 %. Adding 20 %(v/v) DMSO resulted in a two-fold increase in Lip_2 activity with an ee of -25.04 %. A four-fold increase in the reaction volume led to a 10 % decrease in Lip_2 activity with an ee of -13.96 %.

Keywords

Enantiomeric resolution; Lipases; Biotransformation; Kinetic resolution

Resumo

As propriedades biológicas e aplicações de compostos quirais dependem da sua configuração. Biotransformações permitem a enantioseparação de compostos quirais sob condições suaves e a utilização do rótulo *natural*.

A enzima Lip_1 hidrolisava seletivamente *(S)*-metil 2-fenilpropanoato (MPP). Sob as concentrações apropriadas de enzima e substrato, a velocidade de hidrólise do substrato foi 425.6 μmol MPP/mg Lip_1.h com excesso enantiomérico (ee) de 53.7 %. A atividade específica de Lip_1 não mostrou variação significativa com a adição de metanol ou sulfóxido de dimetilo (DMSO) ao meio reacional até uma concentração de 20 %(v/v). A 30 %(v/v) de metanol ou DMSO, a atividade de Lip_1 foi reduzida em 3.14 vezes e aumentada em 6 % com ee de 6.88 % e 55.7 %, respetivamente. Um aumento de quatro vezes do volume reacional levou a uma diminuição de 45 % na atividade de Lip_1 e a um ee de 28.2 %.

Verificou-se que a enzima Lip_2 hidrolisava seletivamente *(R)*-MPP. Sob as concentrações apropriadas de enzima e substrato, a velocidade de hidrólise do substrato foi 0.344 μmol MPP/mg Lip_2.h com um ee de -10.9 %. A adição de 20 %(v/v) de metanol ao sistema reacional levou a um aumento de 37 % da atividade específica de Lip_2 com um ee de 6.23. A adição de 20 %(v/v) DMSO resultou na duplicação da atividade de Lip_2 com um ee de -25.04 %. Um aumento de quatro vezes do volume reacional levou a uma diminuição de 10 % da atividade de Lip_2 com um ee de -13.96 %.

Palavras Chave

Resolução enantiomérica; Lipases; Biotransformação; Resolução cinética

Contents

List of Figures

List of Tables

Acronyms

***(R)*-BINAP**	(*R*)-(+)-(1,1'-Binaphtalene-2,2'-diyl)bis(diphenylphosphine)
BPPM	(-)-*(2S,4S)*-t-butyl-4-(diphenylphospino)-2-(diphenylphosphinomethyl)-1-pyrrolidinecarboxylate
CSP	Chiral stationary phase
DMSO	Dimethyl sulfoxide
DO	Dissolved oxygen
ee	Enantiomeric excess
EMA	European Medicines Agency
EU	European Union
FDA	Food and Drug Administration
GC	Gas chromatography
HFCS	High fructose corn syrup
HPLC	High performance liquid chromatography
INMARE	Industrial Applications of Marine Enzymes
IUBMB	International Union of Biochemistry and Molecular Biology
IUPAC	International Union of Pure and Applied Chemistry
LB	Lysogeny broth
MPP	Methyl 2-phenylpropanoate
NSAID	Nonsteroidal anti-inflamatory drug
OD	Optical density
***p*-NPB**	4-Nitrophenyl butyrate
PTFE	Poly(tetrafluroethylene)
THF	Tetrahydrofuran

Tris Tris(hydroxymethyl) aminomethane

1

Introduction

Contents

Nature is chiral. When observed, both enantiomers are not equally as common in nature; this is true for all living material be it vegetable or animal and even for minerals. Since chiral molecules have different interactions with other chiral molecules, pure enantiomers and racemic mixtures have different biological properties such as flavour, odour and pharmacodynamics which means enantiopurity is of great importance to industries such as food, cosmetic or pharmaceutical. Enzymes are nature's catalysts, making them the ideal way to obtain chiral products.

1.1 Biocatalysis

Biocatalysis is the use of living systems or their components to increase the rate of a chemical reaction. Most biocatalysts are enzymes. Enzyme reactions can be described by Equation 1.1, in which E, S and P represent the enzyme, substrate and product respectively, and ES and EP represent the respective complexes.

$$\mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{ES} \rightleftharpoons \mathrm{EP} \rightleftharpoons \mathrm{E} + \mathrm{P} \tag{1.1}$$

The catalytic effect of enzymes stems from the stabilization of the transition states formed during the course of the reaction which causes the reaction to have a lower activation energy than the non-catalyzed reaction and thus a higher rate, as shown in Figure 1.1. This was first hypothesized by Linus Pauling in 1948 [1].

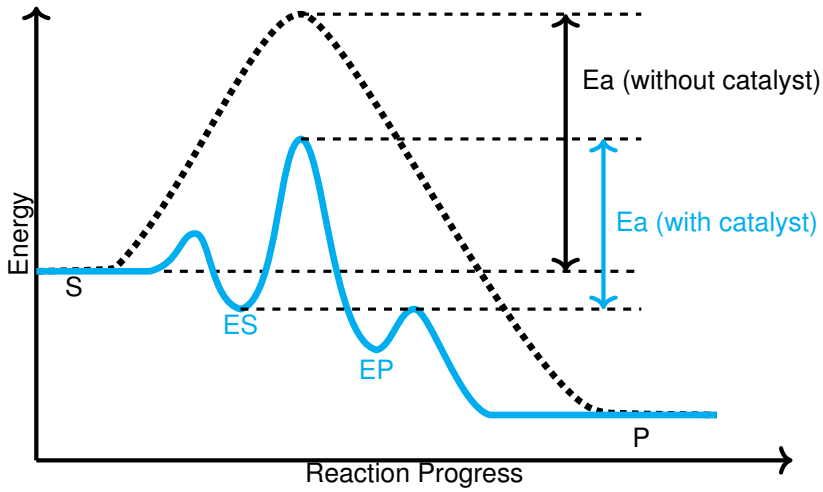


Figure 1.1: Comparison between the Gibbs free energy profiles of a non-catalyzed reaction (dotted black line) and an enzyme catalyzed reaction (cyan) with the same substrate and final products. The required activation energy (Ea) for the catalyzed reaction is lower than the activation energy for the non-catalyzed reaction which leads to a higher reaction rate. Adapted from [2].

1.1.1 A brief history on enzymes

Enzymes have been used for thousands of years to produce bread, wine and beer. Until the nineteenth century it was believed that processes such as the souring of milk and the fermentation of sugar into alcohol required a living organism [3,4]. In 1833, the French chemists Anselme Payen and Jean-François Persoz used alcohol to precipitate *something* from malt extract. This *something* would convert starch into sugar and was inactivated by heat. They called their discovery diastase, from the greek $\delta\iota\alpha\sigma\tau\alpha\sigma\iota\zeta$ (*diastasis* - separation) [5].

In 1836, the second enzyme was extracted from gastric fluids by the German chemist Theodor Schwann and was given the name pepsin [6]. These and other preparations were given the name ferments. This name would eventually be replaced by the name enzyme, which was proposed by the first time in 1877 by the German chemist Wilhelm Kühne [7].

In 1897, the German chemist Eduard Buchner demonstrated by the first time that it was possible to ferment sugar without the presence of living cells using yeast extract [8]. This earned him the Nobel prize in chemistry in 1907.

In 1926 the American chemist James Sumner managed to purify and crystallize the enzyme urease from the plant *Canavalia ensiformis*, from this point it was easy to discover it's structure and other properties, thus arriving at the conclusion that the enzyme urease was a protein. He earned the Nobel prize in chemistry in 1946 for this discovery [9].

Nowadays we know that enzymes are paramount in cellular bioprocesses and due to their catalytic power and specificity they are also used in several industrial processes.

1.1.2 Enzyme nomenclature

Initially enzymes were classified through their substrate and the suffix -ase, such as lactase, which catalyses the hydrolysis of lactose into its constituent monomers (shown in Equation 1.2). However there are several exceptions to this rule such as tripsin which doesn't have the suffix -ase and catalase which hydrolyses hydrogen peroxide into water and oxygen (shown in Equation 1.3).

$$\underset{\text{Lactose}}{\mathrm{C_{12}H_{22}O_{11}}} + \mathrm{H_2O} \xrightarrow{\text{Lactase}} \underset{\text{Glucose}}{\mathrm{C_6H_{12}O_6}} + \underset{\text{Galactose}}{\mathrm{C_6H_{12}O_6}} \tag{1.2}$$

$$2\,\mathrm{H_2O_2} \xrightarrow{\text{Catalase}} 2\,\mathrm{H_2} + 2\,\mathrm{O_2} \tag{1.3}$$

Due to the rapidly growing number of identified enzymes and the lack of consistency in their nomenclature it was necessary to develop a naming system that was able to systematically classify enzymes. The International Union of Biochemistry (now International Union of Biochemistry and Molecular Biol-

ogy (IUBMB)) published a report with the guidelines of the currently accepted system [10]. This report was revised several times and an electronic version mantained by the IUBMB and International Union of Pure and Applied Chemistry (IUPAC) is freely available online [11]. Enzymes were assigned a code number consisting of four numbers separated by dots. They are divided in seven major classes, and the first digit of the code number shows which major class an enzyme belongs to, as shown in Table 1.1.

Table 1.1: The seven major classes in enzyme nomenclature. Adapted from [3, 11–13].

First digit	Class	Type of reaction catalyzed
1	Oxidoredutases	Oxidation and reduction reactions
2	Transferases	Transfer of a chemical group between two molecules
3	Hydrolases	Hydrolysis reactions
4	Lyases	Addition of groups to double bonds or formation of double bonds by removal of groups
5	Isomerase	Isomerization reactions
6	Ligases	Joining of two molecules coupled with the breakdown of a small chemical group
7	Translocases	Catalysing the movement of ions or molecules across membranes or their separation within membranes

1.1.3 Enzymes in the industry

Enzymes have multiple different uses in varied industries such as biofuel, pharmaceutical and fine and bulk chemical manufacturing (Table 1.2).

Table 1.2: Example uses of enzymes across different industries [14–19].

Industry	Enzyme	Example application
Biofuel	Lipase	Catalyzing Biodiesel Synthesis [14]
	Cellulase	Catalyzing the conversion of cellulose into glucose as a part of simultaneous saccharification and fermention for the production of bioethanol [15]
Pharmaceutical	Alcohol Dehydrogenase	Synthesis of *(S)*-3,5-bistrifluoromethylphenyl (an important intermediate in the synthesis of NK_1 receptor antagonists) [16]
	Deoxyribose-5-phosphate aldolase	Synthesis of the side-chain of statin-type cholesterol drugs [17]
Fine and Bulk Chemical	Nitrile Hydratase	Production of acrylamide from acrylonitrile [18]
	Glucose Isomerase	Production of the sweetener High fructose corn syrup (HFCS) from glucose [19]

The use of biocatalysis in industry has been growing due to the myriad of advantages they present over traditional chemical catalysts. A comparison between the two is presented on Table 1.3.

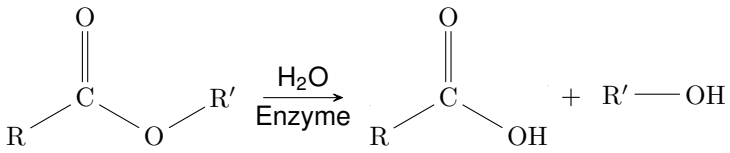
Table 1.3: Main differences between enzymes and chemical catalysts. Adapted from [20].

	Enzymes	Traditional catalysts
Environmental impact	Renewable and biodegradable	Metals can be scarce and/or toxic
Reaction Conditions	Mild - physiological pH, low temperature and atmospheric pressure	Usually high temperature and pressure to speed up the reaction. Acid or basic catalysis requires extreme pH levels
Downstream	Relatively simple	Removing trace elements of some metals to the required extent can be cost prohibitive
Specificity	Very high	Low
Stability	Variable	High
Cost	Variable	Variable

The main advantages of enzyme catalysis are the mild reaction conditions which, aside from the obvious reduced energy costs, the equipment itself is also cheaper; and the specificity they allow. Not only can enzymes be highly specific to the desired substrate, they can also be stereospecific which adds great value to the product, since enantiomerically pure compounds are usually expensive because of their difficulty to synthesize using traditional catalysts [20]. More on this topic is explained in Section 1.2.

1.1.4 Ester hydrolysis

Carboxilic ester hydrolysis reactions (Equation 1.4) are catalysed by carboxilic ester hydrolases (EC 3.1.1.X) [13]. Up to the writing of this work there is not a scientific consensus on the distinction between esterases (carboxylesterases - EC 3.1.1.1) and lipases (tryacylglycerol lipases - EC 3.1.1.3). Initially they were distinguished by their activity on the oil-water interface, in which the lipases had increased activity. However *Candida sp.* lipases were found with low activity in triglyceride hydrolysis [21] and thus ”interfacial activation” was found to be an unsuitable criterion [22]. The presence of a ”lid” in the active site was also proposed as an explanation for the increased activity in interfaces (which supposedly kept the solvent out and would undergo a change in a lipid-water interface) but it was also disregarded [22]. The distinction later moved to hydrolytic activity: esterases showed higher activity on the hydrolysis of shorter carboxyl esters chains while lipases showed higher activity for longer chains [23]. More recently a distinction based on the polarity of the protein surface has also been proposed [24].



(1.4)

Like many hydrolases lipases, and esterases have been found to have a α/β hydrolase fold. This structure is cannonically composed by a sheet of 8 β-sheets connected by α-helices (Figure 1.2). This structure is part of the core of the enzyme and contains the catalytic triad. It's presence across the varied hydrolases indicates a divergence from a common ancestor [25]. Recently slight variations of this structure have been found [26].

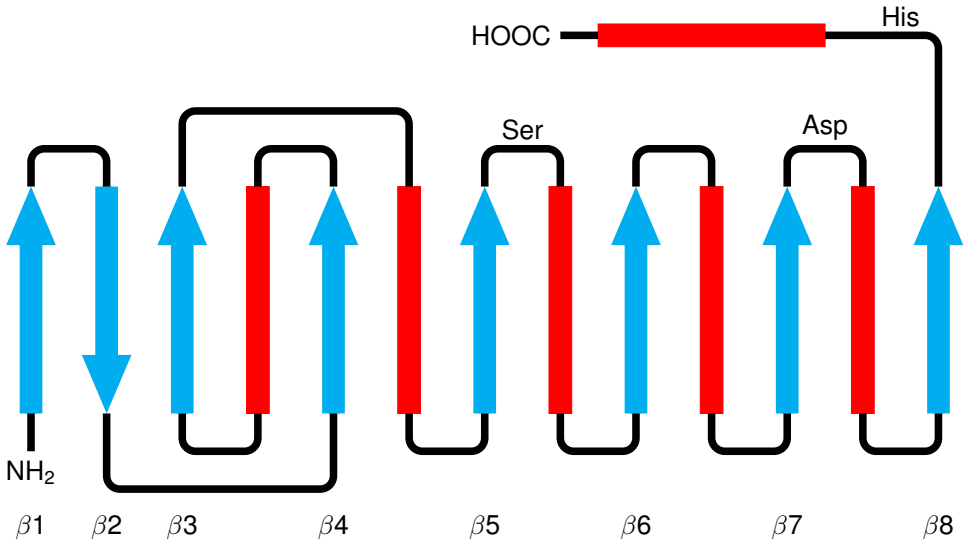


Figure 1.2: Scheme of the cannonical α/β hydrolase fold showing the α-helices (red), the β-sheets (blue) and the catalytic triad. Adapted from [27].

The enzyme's catalytic ability stems from three amino acid residues known as the catalytic triad. This triad is composed by a serine residue which acts as a nucleophile, an histidine residue which acts as a base and an aspartate residue which acts as an acid [28]. Cysteine and aspartate have also been found to act as nucleophiles while glutamate has also been found to act as the acid [29].

The currently accepted enzyme catalysed ester hydrolysis mechanism (Figure 1.3) starts with the ester binding to the active site of the enzyme (two main chain amide N-H, known as the oxyanion hole). The enzyme's catalytic serine residue (nucleophile) is activated by the aspartate and histidine residues. Attack of the serine residue to the ester forms an oxyanion. In this step the serine residue is deprotonated with the histidine residue acting as a base. The oxyanion is stabilized by multiple hydrogen bonds. Reforming of the double bond leads to the alcohol release and the formation of the acyl enzyme intermediate. In this step histidine acts as an acid being deprotonated while the alcohol is protonated. After this a water molecule acts as the nucleophile and another oxyanion intermediate is

formed. Reformation of the double bond allows the acid to leave and the serine residue is reprotonated [27, 30].

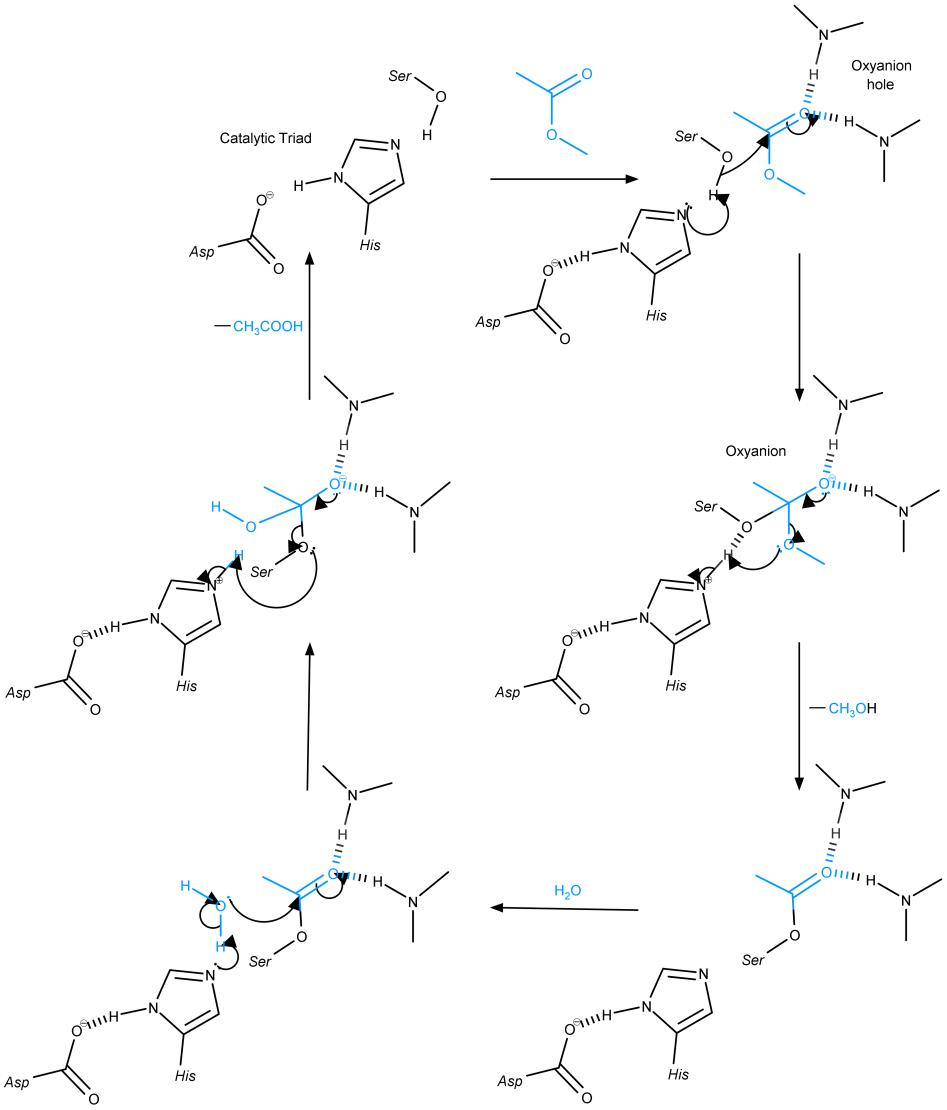


Figure 1.3: Cannonical enzyme catalysed ester hydrolysis mechanism. Reaction scheme for the hydrolysis of methyl ethanoate.

If in thermodinamically favorable conditions, lipases have been found to also catalyse esterification and transesterification reactions [30, 31].

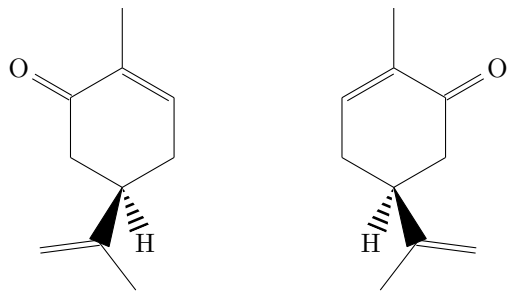
Due to the high substrate variability ester hydrolysing enzymes have a wide range of uses, such as in the food industry, where they can modify fats and oils and act as emulsifiers [32, 33]; in the synthesis of fine chemicals [34]; as detergents [35]; in bioremediation of waste [36] and in the production of biodiesel [37]. Several commercial applications of these enzymes are available on Table 1.4.

Table 1.4: Industrial applications of ester hydrolysis and some patented examples [38–47]. † - Novozym 435 is the most widely used commercial enzyme in industry and academia. Novozym 435 is the lipase B from *Candida antarctica* immobilized on a Lewatit VP OC 1600 resin. This resin is a macroporous support formed by poly(methyl methacrylate) crosslinked with divinylbenzene [48].

Industry	Enzyme	Application	Patent	Reference
Food	Novozym 435† Lipase from *Aspergillus niger*	Lipid modification in oils, milk and cheese to change their flavour and rheological properties	US 20040033571 PT 102638 US 3973042	[38] [39] [40]
	Lipase from *Fusarium venenatum*	Improve properties of dough while baking	US 20030180418 EP 1586240	[41] [42]
Fine chemical	Lipase from *Carica papaya*	Enantiomeric resolution of esters and thioesters	US 20060003428	[43]
	Lipase from *Candida cylindracea*	Preparation of enantiopure α-arylalcanoic acids	US 4762793	[44]
Energy	Lipase from *Candida rugosa*	Biodiesel production	JP 2016116535	[45]
Cleaning	Lipase from *Pseudomonas sp.* *Humicola sp.*	Detergent	US 6017866 WO 9708281	[46] [47]

1.2 Chirality

Molecules that share the same number of each of its constituent atoms but are different are called isomers. Isomers that have their atoms bonded in the same sequence, but with a different spacial arrangement are called stereoisomers. Two steroisomers that form non-superimposable mirror images are called enantiomers (Figure 1.4). Non-mirror imaged stereoisomers are called diastereomers. Stereoisomers are said to be *chiral*. On organic compounds the source of chirality is most often a carbon atom bonded to four different atoms (or groups of atoms). Most biomolecules are chiral since saccharides, nucleotides and all aminoacids (except glycine) show chirality.



(R)-Carvone *(S)*-Carvone

Figure 1.4: Chemical stuctures of the *R*- and *S*-enantiomers of carvone.

Enantiomers have the same physical properties such has molecular weight, boiling or melting point and density. However, they have two major diferences: their rotation of polarized light (which is opposite for each enantiomer) and their interaction with other chiral molecules. Since most biomolecules are chiral, enantiomers can have vastly different biological properties [49]. For example (R)-carvone has a spearmint aroma while (S)-carvone has a caraway aroma [50].

Because of the difference in biological properties between enantiomers, the usage of enantiomerically pure components is important across many industries, especially in the pharmaceutical industry where it is paramount because the non-therapeutic enantiomer can have unwanted side-effects. The thalidomide disaster in the late 1950s is the most well known example of this, being responsible for limb malformations across several countries in tens of thousands of victims. For thalidomide the *R*-enantiomer is therapeutic while the *S*-enantiomer is teratogenic [51, 52]. This disaster led to increased drug regulations across the globe such as the Directive 65/65/EEC in 1965 in the European Economic Community (now European Union (EU)) and the Kefauver-Harris drug amendments in 1962 in the United States of America [53, 54]. In 1994 guidelines for the development of chiral drugs outlining the necessity of testing each enantiomer were put into effect in the EU by the European Medicines Agency (EMA). Similar guidelines were put into place by the Food and Drug Administration (FDA) in the United States of America in 1992 [55, 56].

1.3 Enantiomeric resolution

As previously explained in Section 1.2 there is a regulatory mandated necessity of obtaining enantiomerically pure compounds in the pharmaceutical industry which leads to great interest in this field. Although the easiest way to obtain an enatiopure compound is to isolate it directly from nature, it is often the case that direct isolation from a natural source is impossible, and a mixture must be resolved. Chromatographic methods and kinetic resolutions are the most widely used methods.

1.3.1 Chromatographic methods

Chromatographic techniques are the most widely used in enantiomeric separation.

1.3.1.A High performance liquid chromatography

High performance liquid chromatography (HPLC) has been used to separate enantiomers through chiral phases (direct separation) and through chiral derivatization reagents (indirect separation). Direct separation using a chiral stationary phase (CSP) is the most convenient, and easiest to scale up, however it requires different columns when meaning to solve a variety of mixtures. Indirect separation using a

derivatization reagent circumvents the need for a chiral stationary phase but the derivatization reaction is another step which can involve unwanted side reactions or even racemization [57, 58].

A chiral HPLC system with a ChiralPak AD CSP has been scaled up to resolve 1.04 t/year of a racemic mixture of an active pharmaceutical ingredient, with an estimated cost of 40 €/kg [59]. The most widely used derivatization agent is *o*-phthalaldehyde [60] due to its use in chiral separation of amines in the presence of thiols and fluorescent properties [61].

1.3.1.B Gas chromatography

There are three major types of CSP used in chiral gas chromatography (GC): amino acid derivatives, chiral metal coordination compounds and cyclodextrin derivatives. Chirasil-val columns were the first amino acid based columns developed and quickly showed great results in amino acid chiral resolution [62, 63]. (-)-menthyl chloroformate is one of the most widely used derivatization agents due to its use in the chiral resolution of α-hydroxy acids, amino acids and alcohols [64]. *(S)*-amphetamine and *(R)*-amphetamine have been used to derivatize profens [65].

Chiral GC is not a popular separation method at an industrial scale, being more widely used for analytical purposes.

1.3.2 Enzymatic kinetic resolution

Kinetic resolution is a reaction on which one of the enantiomers in a mixture is more readily transformed into a given product than the other. When using enzyme catalysts, ideally only one of the enantiomers reacts, and the reaction can reach total conversion. However since the reaction progresses through time, often is the case that the enantioselectivity decreases due to an increasing difference between the concentrations of both enantiomers [66]. Among the vast amount of enzymes, lipases have been the most widely reported in kinetic resolution due to their availability and wide substrate range [66].

If the chiral product is what is desired (as opposed to the enantiopure form of the substrate) the *in situ* racemization of the substrate can be employed. This process is called dynamic kinetic resolution. For an efficient dynamic kinetic resolution, the kinetic resolution step needs to be irreversible and highly selective, and the racemization needs to have a reaction rate at least equal to the resolution rate [67].

Several examples of enzyme-catalyzed kinetic resolutions are available on Table 1.5.

1.4 Asymmetric synthesis

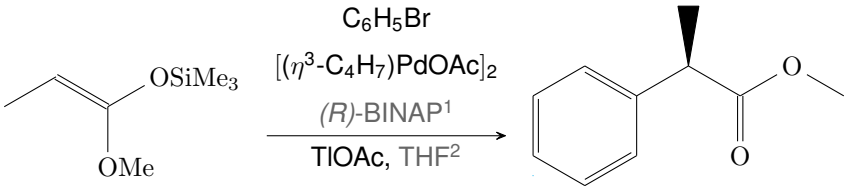
Ideally, the second best way to procure an enantiopure compound (after direct isolation from a natural source) is through asymmetrical synthesis, however good asymmetric reaction systems can be very hard

Table 1.5: Several examples of kinetic resolutions using enzymes as catalysts. †Subtilisin Carlsberg is a commercially available bacterial protease obtained from the fermentation of *Bacillus licheniformis* [68].

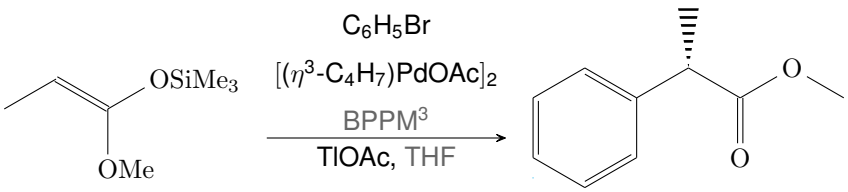
Enzyme	Application	Reference
Lipase B from *Candida antarctica*	Resolution of 2-octanol (through esterification) to >99.9 % to enantiomeric excess (ee) (alcohol) and 88.6 % ee (ester)	[69]
Subtilisin Carlsberg†	Resolution of 1-phenylethanol (through acylation) to 84 % ee	[70]
ω-transaminase from *Bacillus thuringiensis* JS64	Resolution of α-methylbenzylamine up to 95 % ee	[71]
γ-lactamase from *Rhodococcus equi*	Resolution of 2-azabicyclo[2.2.l]hept-5-en-3-one up to 98 % ee	[72]

to find since the necessary knowledge may not yet exist, or the necessary catalyst can be prohibitively expensive or non-existent altogether, and even then the product may not have the necessary ee for the necessary purpose requiring a resolution step nonetheless [73].

A method for the asymmetric synthesis of methyl 2-phenylpropanoate (MPP) was reported by Galarini and colleagues through the asymmetric α-arylation of a silyl ketene acetal using a palladium catalyst. Pathways for both *(R)*-MPP (Equation 1.5) and *(S)*-MPP (Equation 1.6) were reported [74].



(1.5)



(1.6)

The reported results (yield and ee) are represented on Table 1.6. The reported values for ee are too low for pharmaceutical use without posterior resolution.

Table 1.6: Reported yield and ee for the asymmetric synthesis of MPP by Galarini and colleagues. Yields are referred to C_6H_5Br [74].

MPP enantiomer	Yield (%)	ee (%)
(R)-MPP	55	44
(S)-MPP	68	54

[1] (*R*)-(+)-(1,1'-Binaphtalene-2,2'-diyl)bis(diphenylphosphine) (*(R)*-BINAP)
[2] Tetrahydrofuran (THF)
[3] (-)-*(2S,4S)*-t-butyl-4-(diphenylphospino)-2-(diphenylphosphinomethyl)-1-pyrrolidinecarboxylate (BPPM)

1.5 Rationale

The demand for cleaner and more sustainable industrial processes is driving the demand for industrially useful biocatalysts. It's relatively simple to isolate new enzymes from microorganisms, however most enzymes are not commercially significant. The process to identify industrially viable enzymes is long and has a very low success rate. The Industrial Applications of Marine Enzymes (INMARE) (grant agreement number 634486) European project brought together industry and academia partners to isolate new and useful enzymes from the ocean (http://www.inmare-h2020.eu/). The project succeeded in decreasing the average time frame to develop new industrially relevant enzymes from seven to three years.

The INMARE industry partners were interested in immobilized enzymes that were able to catalyse reactions with several different substrates, so INMARE partners focused their attention on finding promiscuous enzymes. Marine environments were chosen as the source of biocatalysts due to having more microbial species than any other environment on the planet [75].

In the pharmaceutical industry, demonstrating the effect of each of the enantiomers in a chiral drug is a regulatory necessity, which makes enantioseparation of extreme importance. The industry partners of the INMARE project provided MPP as a model test substrate for ester hydrolysis reactions due to the small size of one of the chemical groups bound to the chiral carbon (which makes asymmetric synthesis more difficult) and to its similarity to profens (2-arylpropionic acids) which are an important class of nonsteroidal anti-inflamatory drugs (NSAIDs).

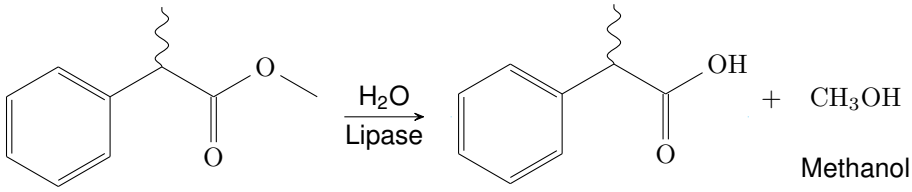
The INMARE academia partners provided the biocatalysts analyzed in this work. The enzyme Lip_1 was identified and produced by the group of Dr. Manuel Ferrer (CSIC, Spain) and the enzyme Lip_2 was identified and provided by the group of Professor Karl-Erich Jaeger (Heinrich Heine University of Düsseldorf, Germany).

1.5.1 Objectives

The objective of this work was to evaluate the catalytic potential of the enzymes Lip_1 and Lip_2 in the enantioselective hydrolysis of MPP and the optimization of the reaction system for each enzyme taking into account process conditions, according to INMARE requirements. The scale-up of the reaction system was also an important part of this work.

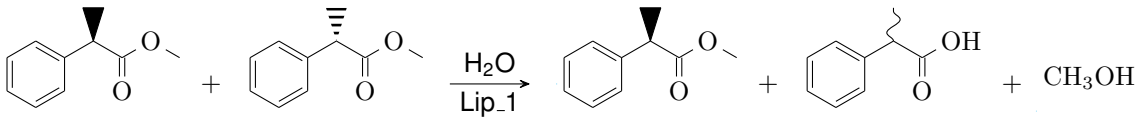
1.6 Enzymatic kinetic resolution of methyl 2-phenylpropanoate

The hydrolytic activity of the lipases Lip_1 and Lip_2 on MPP (Equation 1.7) was studied in order to evaluate their enantioselective capability.

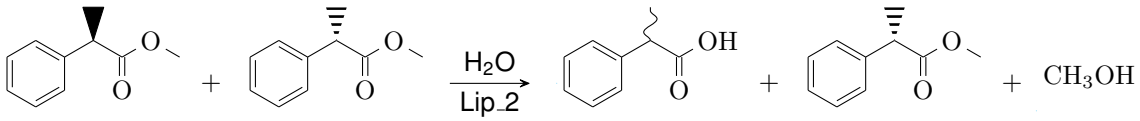


MPP 2-Phenylpropionic acid (1.7)

The lipase Lip_1 was found to selectively hydrolyse the *S*-enantiomer of MPP (Equation 1.8), while the lipase Lip_2 was found to selectively hydrolyse the *R*-enantiomer of MPP (Equation 1.9).



(R)-Methyl 2-phenylpropanoate + *(S)*-Methyl 2-phenylpropanoate → *(R)*-Methyl 2-phenylpropanoate + 2-Phenylpropionic acid + Methanol (1.8)



(R)-Methyl 2-phenylpropanoate + *(S)*-Methyl 2-phenylpropanoate → 2-Phenylpropionic acid + *(S)*-Methyl 2-phenylpropanoate + Methanol (1.9)

2

Materials and Methods

Contents

2.1 Reagents

MPP was kindly provided by a partner of the EU funded project INMARE. It was provided as a mixture of the *S* and *R* enantiomers at 45.1 % and 54.9 % (molar), respectively (ee of 9.8 %). 4-Nitrophenyl butyrate (*p*-NPB) ($\geq$98 %) and D-(+)-Glucose ($\geq$99.5 %) were purchased from Sigma (St. Louis, MO, USA), tris(hydroxymethyl) aminomethane (Tris) was purchased from Eurobio Scientific (Paris, France), methanol (HPLC grade), acetonitrile (HPLC grade), dimethyl sulfoxide (DMSO) ($\geq$99.7 %), and glycerol (99.96 %) were purchased from Fisher Scientific (Waltham, MA, USA), sodium chloride ($\geq$95 %) and hydrochloric acid ($\geq$37 %) were purchased from Honeywell Fluka™ (Muskegon, MI, USA) , *n*-hexane (HPLC grade) was purchased from Panreac AppliChem (Darmstadt, Germany), diethyl methylmalonate ($>$98 %) was purchased from Tokyo Chemical Industry (Tokyo, Japan), Na_2HPO_4 ($>$ 99 %) was purchased from Merck (Darmsatdt, Germany), K_2HPO_4 was purchased from Panreac Quimica SA (Barcelona, Spain), tryptone was purchased from ORGANOTECHNIE (La Courneuve, France), yeast extract was purchased from Liofilchem (Roseto degli Abruzzi, Italy), lactose monohydrate was purchased from Merck (Darmstadt, Germany), lysogeny broth (LB) and LB Agar were purchased from Nzytech (Lisbon, Portugal). All aqueous solutions were prepared using Milli-Q® water except LB and LB agar media which were prepared using distilled water.

2.2 Enzymes

The enzyme Lip_1 used in this work was kindly provided by Dr. Manuel Ferrer (CSIC, Spain), parnter of the INMARE project. For reasons of confidentiality the real name of this enzyme will not be revealed. This enzyme was provided inside lyophilized cells.

2.3 Bacterial strains

The bacterial strain *E. coli* BL21 (DE3) pET22b_Lip_2 was kindly provided by Professor Karl-Erich Jaeger (Heinrich Heine University of Düsseldorf, Germany), partner of the INMARE project. For reasons of confidentiality the name of the plasmid of this strain will not be revealed. This *E. coli* strain has been genetically modified to overexpress the enzyme Lip_2. The bacterium stock was stored at -80 °C in 20 % glycerol. Fresh cultures were prepared in Petri dishes with LB agar medium and were stored at 4 °C. New cultures were prepared each month.

2.4 Bacterial growth

The cellular growths were performed in Erlenmeyer flasks. The composition of the growth media is available at Appendix B - Tables B.1 and B.2. A pre-inoculum was done overnight on 100 mL flasks containing 20 mL of pre-inoculum medium at 30 °C and 160 rpm in an Agitorb 200 incubator (Aralab, Portugal). The pre-inoculum was used to inoculate 500 mL flasks containing 100 mL of induction medium at a starting optical density $(OD)_{580}$ of 0.1. Cultures grew overnight at 30 °C and 160 rpm. After cellular growth the medium was centrifuged on a RC-6 centrifuge from Sorvall at 10,000xg for 10 min. The cell pellet was harvested and softly washed with 5 mL of Tris-HCl 100 mM pH 8.0 buffer and subsequently centrifuged again under the same conditions on a 5810 R centrifuge (Eppendorf, Hamburg, Germany). This pellet was harvested and stored at -20 °C until further use.

2.4.1 Oxygen consumption

The online analysis of the oxygen concentration during cellular growth was performed using *OxoDish® OD24* microtiter plates by a SDR *SensorDish® Reader* (PreSens Precision Sensing GmbH, Regensburg, Germany). Each of the wells contained 2 mL of the growth medium under study and was inoculated in order to obtain a starting OD_{580} of 0.1. The cellular growth was monitored for 4.5 h and the oxygen data was collected every 10 min.

2.5 Enzymatic assays

2.5.1 4-Nitrophenyl butyrate hydrolysis

The reaction was performed inside a 4 mL quartz cuvette (Hellma, Müllheim, Germany) with a 10 mm light path and the absorbance was monitored at 410 nm during 90 s with measurements taken every 10 s using an Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) connected to a computer. To the cuvette, 1470 μL of Tris-HCl 20 mM pH 8.0 buffer and 15 μL of the biocatalyst were added. Temperature control and stirring speed were set to 30 °C and 600 rpm and the cuvette was left to thermally stabilize for 4 min. To start the reaction, 15 μL of a 70 mM solution of *p*-NPB in acetonitrile was added (adapted from [76]). One unit of enzymatic activity - U (*p*-NPB) - was defined as one micromole of *p*-NPB hydrolysed per minute.

2.5.2 Methyl 2-phenylpropanoate hydrolysis

The reaction was performed in VEREX™ vials (23x44 mm, 10 mL, 18 mm screw, round bottom for 1 mL of reaction volume; 23x75 mm, 20 mL, 18 mm screw, round bottom for 2 mL of reaction volume and

28x95 mm, 40 mL, 24 mm screw for 4 mL of reaction volume), closed with VEREX™ screw caps (18 mm or 24 mm with poly(tetrafluroethylene) (PTFE) and silicone septa, respectively) aquired from Phenomex (Torrence, CA, USA) and 12x3 mm magnetic stirring bars (Kartell, Noviglio, Italy), on a Variomag Poly 15 Multi-Point Inductive-Drive Stirrer (Thermo Scientific, Waltham, MA, USA) (data available at Appendix D - Table D.1). Stirring speed was set to 600 rpm. Temperature was controlled by a Certomat® H incubator from B.Braun (Hessen, Germany) and set to 30 °C. The compounds were added in the following order: MPP, cosolvent, Tris-HCl 100 mM pH 8.0, enzyme. Before the enzyme was added the vials were left under the reaction temperature and stirring for 15 min. For reactions over one hour, the vials were pressurized in order to slow down evaporation of MPP. This was done by injecting air with a syringe through the vial caps. On the 10 mL vials, this was done using a 5 mL Injekt® Luer Solo from B. Braun. The 20 mL vials were pressurized using a 10 mL syringe (Luer lock, from Terumo) and the 40 mL vials were pressurized using a 20 mL syringe (Luer Lock, from Fisher). The needles used were Neolus® hypodermic needles 0.5x25 mm from Terumo (Tokyo, Japan).

The reactions were stopped by a liquid-liquid extraction of MPP using 1 mL of an hexane and diethyl methylmalonate solution (330 μL of diethyl methylmalonate in 100 mL of solution) which was added to the reaction medium. This mixture was transferred to a 2 mL Eppendorf tube and shaken for 5 min on a vortex. Reactions with 2 and 4 mL of reaction volume were stopped by adding 2 and 4 mL of the hexane and diethyl methyl malonate solution, respectively. The solutions were transferred to 5 mL Eppendorf tubes and 15 mL Falcon Tubes, respectively. The mixture was then centrifuged at 10,000xg for 10 min on a μSpeedFuge® SFA13K centrifuge (from Savant), for the 2 mL Eppendorf tubes and on a 5810 R centrifuge (from Eppendorf) for the 5 mL Eppendorf tubes and 15 mL Falcon tubes.

2.6 Analytical methods

2.6.1 Biomass determination

Biomass was determined by OD measurements at 580 nm on a Thermo Scientific Multiskan® GO spectrophotometer from Fisher Scientific (Waltham, MA, USA) using a 1.5 mL glass cuvette (Hellma, Müllheim, Germany) with a 10 mm light path. The calibration data is available at Appendix A - Figure A.1.

2.6.2 Methyl 2-phenylpropanoate analysis

MPP concentration in hexane was measured by gas chromatography on a GC-2010 Plus GC from Shimadzu (Kyoto, Japan). This GC was equipped with a flame ionization detector (FID-2010 Plus), an AOC-20i auto injector and an AOC-20s auto sampler. The column used was a 25 m CP-Chirasil-Dex CB with an inner diameter of 0.25 mm and a film thickness of 0.25 μm from Agilent (Santa Clara, CA, USA).

The injection temperature was 250 °C and 1 μL samples were injected in split mode at a 1:100 ratio. The oven temperature was set at 120 °C for 10 min and increased until 170 °C at a rate of 10 °C/min where it was kept for 1 min. Diethyl methylmalonate was used as internal standard. The calibration data is available at Appendix A - Figures A.2 and A.3.

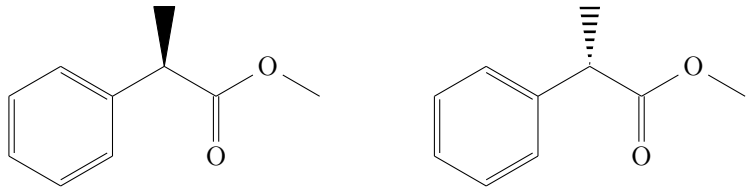
2.7 Enantiomeric Excess

The relative abundance between both enantiomers e_1 and e_2 in a mixture is quantified by the ee, which is defined according to Equation 2.1.

$$\text{ee } (\%) = \frac{[e_1] - [e_2]}{[e_1] + [e_2]} \times 100 \tag{2.1}$$

In this work e_1 represents the *R*-enantiomer, while e_2 represents the *S*-enantiomer of MPP (Figure 2.1), which when computed into Equation 2.1 forms the definition of ee presently used (Equation 2.2).

$$\text{ee } (\%) = \frac{[R] - [S]}{[R] + [S]} \times 100 \tag{2.2}$$

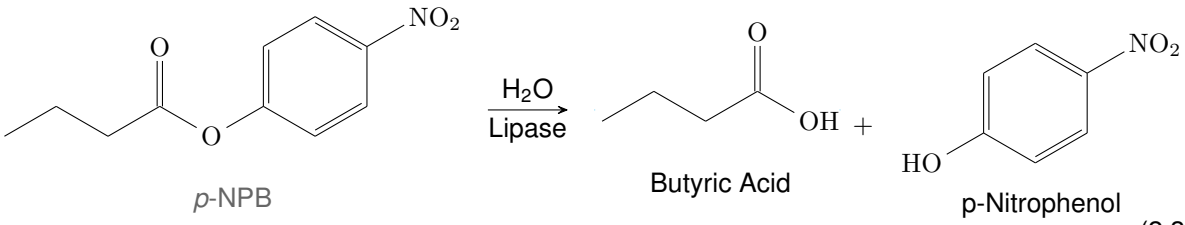


(R)-Methyl 2-phenylpropanoate *(S)*-Methyl 2-phenylpropanoate

Figure 2.1: Chemical structures of the *R* and *S* enantiomers of methyl 2-phenylpropanoate.

2.8 Enzyme quantification

The total amount of enzyme added in each of the MPP hydrolysis assays was quantified through enzymatic activity on *p*-NPB hydrolysis (Equation 2.3). In order to normalize different batches of enzyme in the MPP hydrolysis assays the amount of enzyme added corresponded to a fixed amount of *p*-NPB activity.



p-NPB Butyric Acid p-Nitrophenol (2.3)

The activity for *p*-NPB hydrolysis was determined using the Lambert-Beer law (Equation 2.4), which relates the change in absorbance ($^{dA}/_{dt}$) to the rate of a reaction ($^{dC}/_{dt}$) using the molar absorptivity (ε) and the light path length of the cuvette (l) (Table 2.1).

$$\frac{dA}{dt} = \varepsilon l \frac{dC}{dt} \tag{2.4}$$

Table 2.1: Values for the constants ε and l used in the Lambert-Beer law for the system in use.

Molar absorptivity (ε)	15.4$\times 10^3$ $M^{-1}cm^{-1}$ [77]
Light path length (l)	1 cm

3

Results and discussion

Contents

3.1 Enzymatic characterisation

3.1.1 Esterase activity

Lip_1 cells were found to have a much higher specific esterase activity (1297.3 times) than Lip_2 cells in *p*-NPB hydrolytic activity at 30 °C. Lip_2 is an intracellular enzyme, as expected, since *E. coli* is a very poor secretor of proteins (Table 3.1) [78].

Table 3.1: Specific activity of the enzymes Lip_1, Lip_2 and of the supernatant of the Lip_2 growth medium on p-NPB hydrolysis. † - Activity expressed as U (p-NPB)/μL.

Sample	Specific activity U (*p*-NPB)/g
Lip_1 cells	144×10^{3}
Lip_2 cells	111×10^{0}
Lip_2 growth supernatant	0†

3.1.2 Lip_1 characterisation

3.1.2.A Effect of enzyme concentration on Lip_1 activity

By increasing Lip_1 concentration the ee also increased until the concentration corresponding to 19.5 U (Figure 3.1a). The slight decrease that happened at 26 U was due to a decrease in the total amount of *(S)*-MPP hydrolysed (Figure 3.1c) which was caused by a reduction in the total activity of Lip_1 on this substrate. A plateau was the expected behaviour [79], and there were no supporting arguments found in the literature for this decrease, however similar anecdotes were observed in our laboratory [80]. This is likely due to mass transfer problems caused by the presence of the cells.

The increase of enzyme concentration also led to a decrease of specific activity on the *S*-enantiomer and an increase of the specific activity on the *R*-enantiomer (until 19.5 U - Figure 3.1b). Since the starting substrate concentration was constant the substrate-enzyme ratio decreased with an increase in enzyme concentration which should have led to reduced specific activity, however since there is still an increase in total activity the reaction mixture will shift towards the *R*-enantiomer which led to lower selectivity [81], which in this case was shown by the increase in the *(R)*-MPP specific activity.

Lip_1 concentration corresponding to 13 U (*p*-NPB) was chosen for the following assays because at this concentration the enzyme is the limiting factor and has higher activity than the previous concentrations.

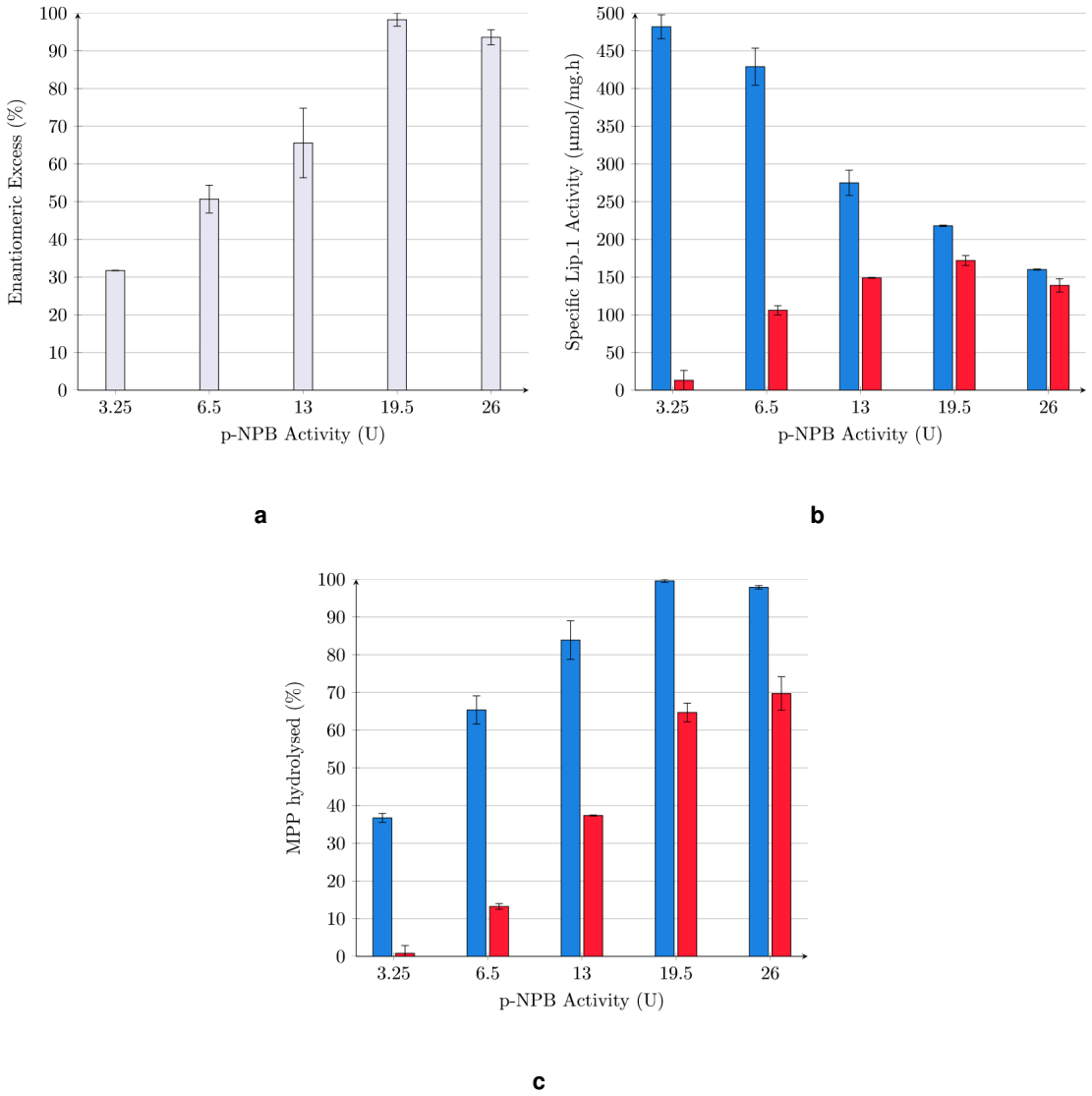


Figure 3.1: Effect of the variation of Lip_1 concentration (quantified through p-NPB activity) on: a the enantiomeric excess of MPP; b specific activity of Lip_1 on the *S* (blue) and *R* (red) enantiomers of MPP; and c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed during the reaction. The initial MPP concentration was 40 mM and the reaction time was 30 min.

3.1.2.B Effect of Substrate concentration on Lip_1 activity

When using a starting MPP concentration of 20 mM enantiomerically pure *(R)*-MPP was achieved (Figure 3.2a) due to complete hydrolysis of *(S)*-MPP (Figure 3.2c). However, 81.8 % of *(R)*-MPP was also hydrolysed resulting in a yield of only 18.2 % on *(R)*-MPP. Increasing the substrate concentration increases Lip_1 activity, and also its selectivity due to increased availability of the preferred *S*-enantiomer of MPP (Figure 3.2b). After 80 mM Lip_1 activity on *(R)*-MPP increases, reducing the selectivity.

MPP concentration of 60 mM should have been chosen as the starting concentration for the following

assays for its balance between conversion and selectivity. However, 40 mM was the concentration chosen due to an error in the earlier analysis of this system.

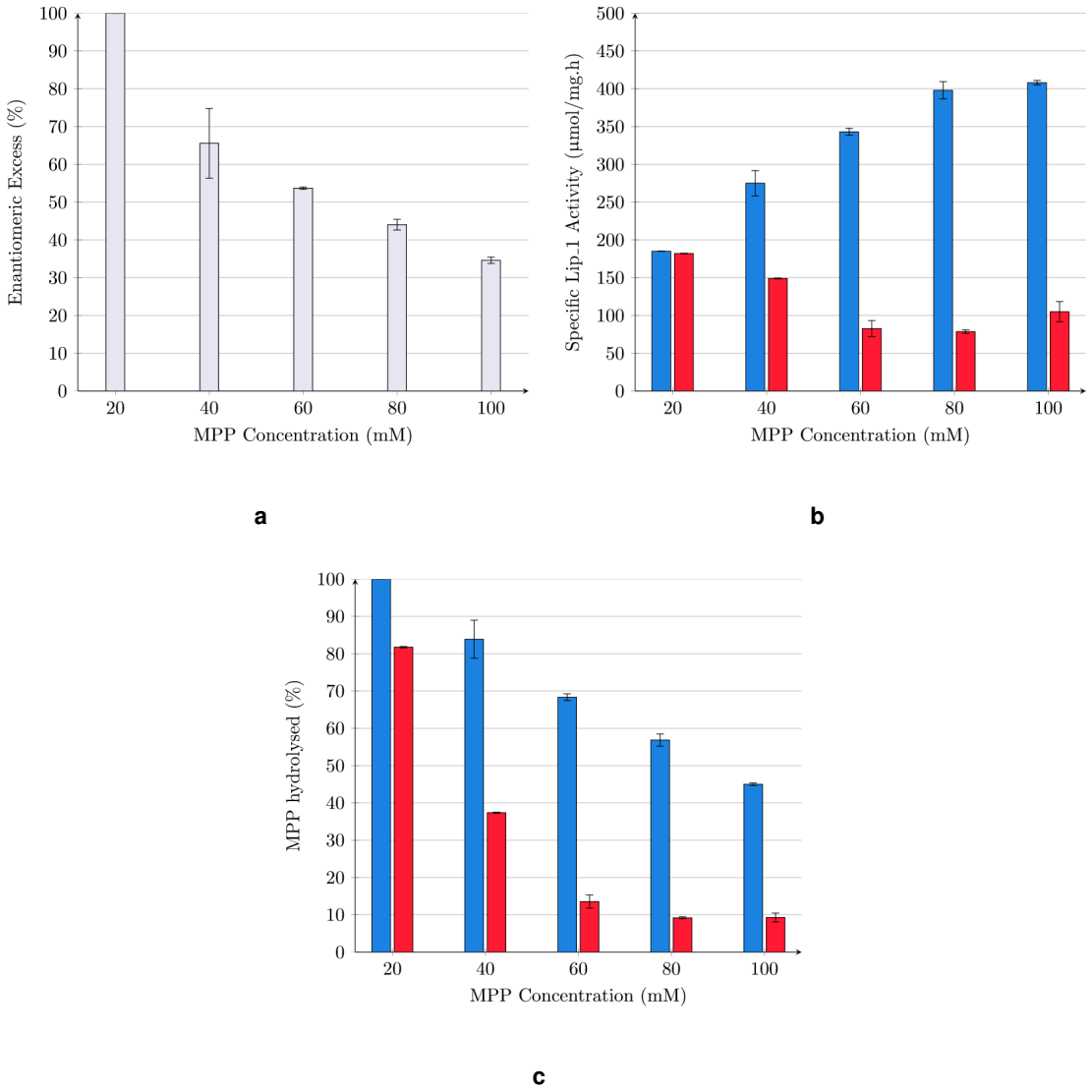


Figure 3.2: Effect of the variation of methyl 2-phenylpropanoate concentration on: a the enantiomeric excess of MPP; b specific activity of Lip_1 on the *S* (blue) and *R* (red) enantiomers of MPP; and c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed during the reaction. Since the *S* enantiomer of MPP was completely hydrolysed when using a starting MPP concentration of 20 mM, the corresponding specific activity is greater than or equal to what is shown. The enzyme concentration added corresponded to 13 U (*p*-NPB) and the reaction time was 30 min.

3.1.2.C Co-solvent effect on Lip_1 activity

Aqueous polar organic solvents have been shown to affect the activity of lipases, due to binding of solvent molecules to a hydrophobic patch in the vicinity of the active site of the enzyme and therefore

changing the active site environment [82]. In this work the effect of methanol and DMSO on the activity of the enzymes was assessed.

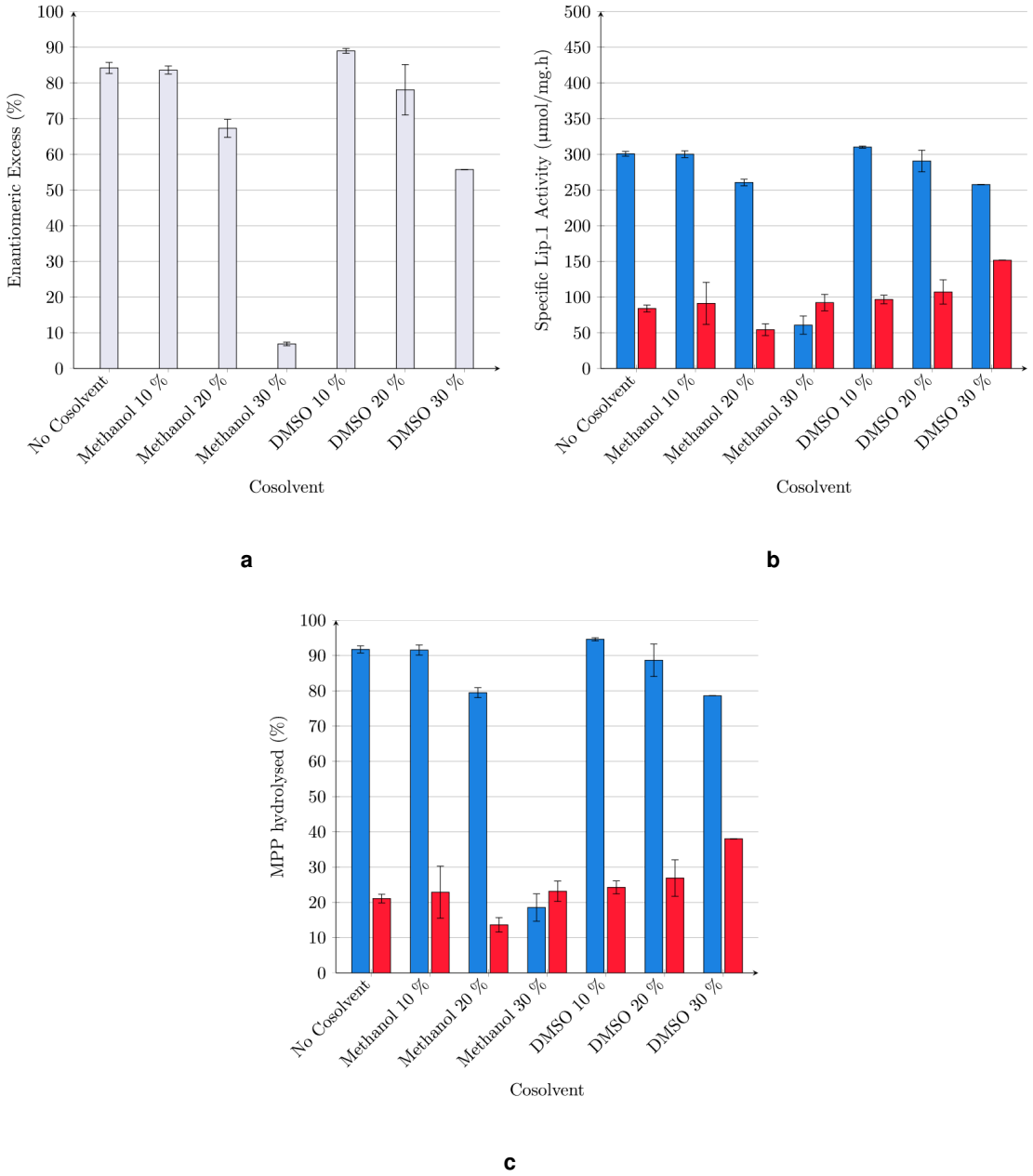


Figure 3.3: Effect of the presence of methanol and DMSO in the reaction media at 10, 20 and 30 %(v/v) on: a the enantiomeric excess of MPP; b specific activity of Lip_1 on the *S* (blue) and *R* (red) enantiomers of MPP; and c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed during the reaction. The enzyme concentration added corresponded to 13 U (*p*-NPB), the initial MPP concentration was 40 mM and the reaction time was 30 min.

Methanol at 10 % (v/v) has no efect on Lip_1 activity, at 20 % it causes a loss of 13.2 % and 40.4 %

of activity on the *S*- and *R*-enantiomers of MPP, at 30 % methanol causes a change in Lip_1 selectivity, making it hydrolyse the *R*-enantiomer faster than the *S*-enantiomer (Figure 3.3b). DMSO has little effect on the activity of Lip_1 up to 20 %, while at 30 % it causes a 14.3 % drop and an 80.4 % increase in the specific activity of Lip_1 in the hydrolysis of the *S*- and *R*-enantiomers of MPP, respectively (Figure 3.3b).

Although when using 20 % DMSO as co-solvent showed the best results, with a slight increase in the conversion of *(S)*-MPP which leads to increased ee (Figures 3.3c and a), none of the tests showed a significant effect on activity to justify the use of solvents. The following assays were done without the usage of any co-solvent.

3.1.2.D Bioreaction run

During the first 15 min of the hydrolysis reaction using a starting MPP concentration of 40 mM and no co-solvent, Lip_1 activity on the *R* enantiomer was 11.2 times lower than Lip_1 activity on the *S* enantiomer (Figure 3.4b). This decreased to 1.8 times at 30 min of reaction time. The decrease with time of the selectivity was expected since the relative abundance between the enantiomers shifts towards the *R* enantiomer (Figure 3.4a). After 60 min of reaction enantiomerically pure *(R)*-MPP was achieved (Figures 3.4c and 3.4d).

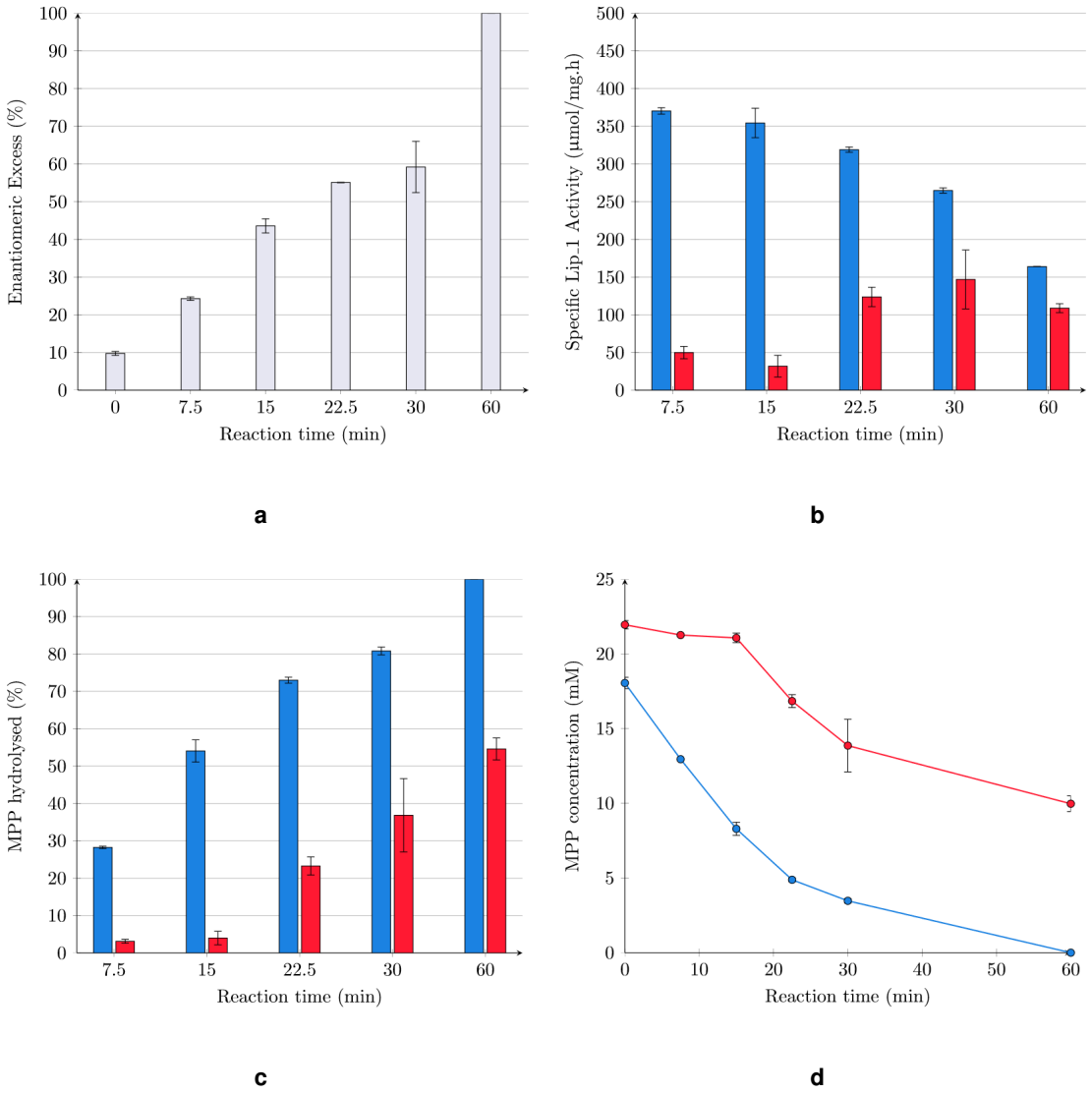


Figure 3.4: Hydrolysis of MPP by Lip_1: a enantiomeric excess of MPP along the reaction progress; b specific activity of Lip_1 on the *S* (blue) and *R* (red) enantiomers of MPP; c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed; and d concentration of the *S* (blue) and *R* (red) enantiomers of MPP over the course of the reaction. Since the *S* enantiomer of MPP was completely hydrolysed after 60 min of reacion time, the corresponding specific activity is greater than or equal to what is shown. The enzyme concentration added corresponded to 13 U (*p*-NPB), the initial MPP concentration was 40 mM and no co-solvent was used.

3.1.2.E Scale-up

When increasing the reaction volume from 1 mL to 4 mL a clear reduction of the selectivity was observed (Figure 3.5). This was due to a large decrease of Lip_1 activity on the *S*-enantiomer (52.6 % from 1 to 4 mL), while the Lip_1 activity on the *R*-enantiomer only slightly decreased (19.2 % from 1 to 4 mL) (Figure 3.5 b), which caused a reduction on the conversion of *(S)*-MPP (Figure 3.5c) and on the enantiomeric

excess (Figure 3.5a). This was due to mass transfer phenomena since the average MPP droplet radius increased with the reaction volume which led to a reduced specific area, which led to worse mass transfer, which reduced the available MPP in contact with the enzyme, which naturally reduced activity and selectivity [83].

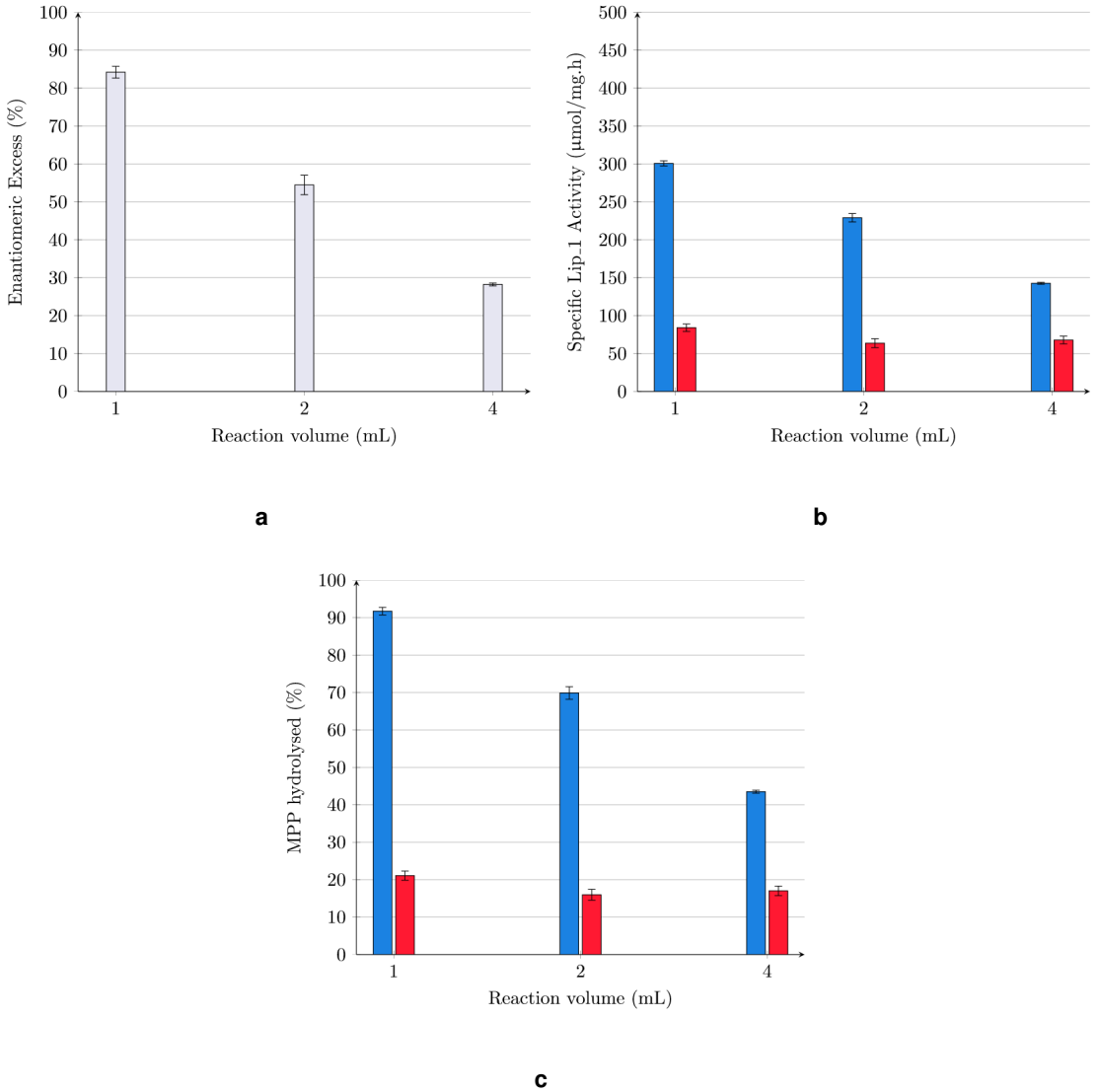


Figure 3.5: Effect of doubling and quadrupling the reaction volume on: a the enantiomeric excess of MPP; b specific activity of Lip_1 on the *S* (blue) and *R* (red) enantiomers of MPP; and c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed during the reaction. The enzyme concentration added corresponded to 13 U (*p*-NPB), the initial MPP concentration was 40 mM, no co-solvent was used and the reaction time was 30 min.

3.1.3 Lip_2 characterisation

3.1.3.A Effect of enzyme concentration on Lip_2 activity

Increasing Lip_2 concentration resulted in an increase in the ee until 4.88 U (Figure 3.6a). The decrease observed at 9.76 U and 14.64 U was caused by a reduction of the total amount of *(R)*-MPP hydrolysed (Figure 3.6a) which in turned was caused by a reduction of total Lip_2 activity on this substrate. As previously explained in section 3.1.2.A, a plateau was the expected behaviour and no support for this phenomenon was found in the literature, however similar anecdotes were available [80].

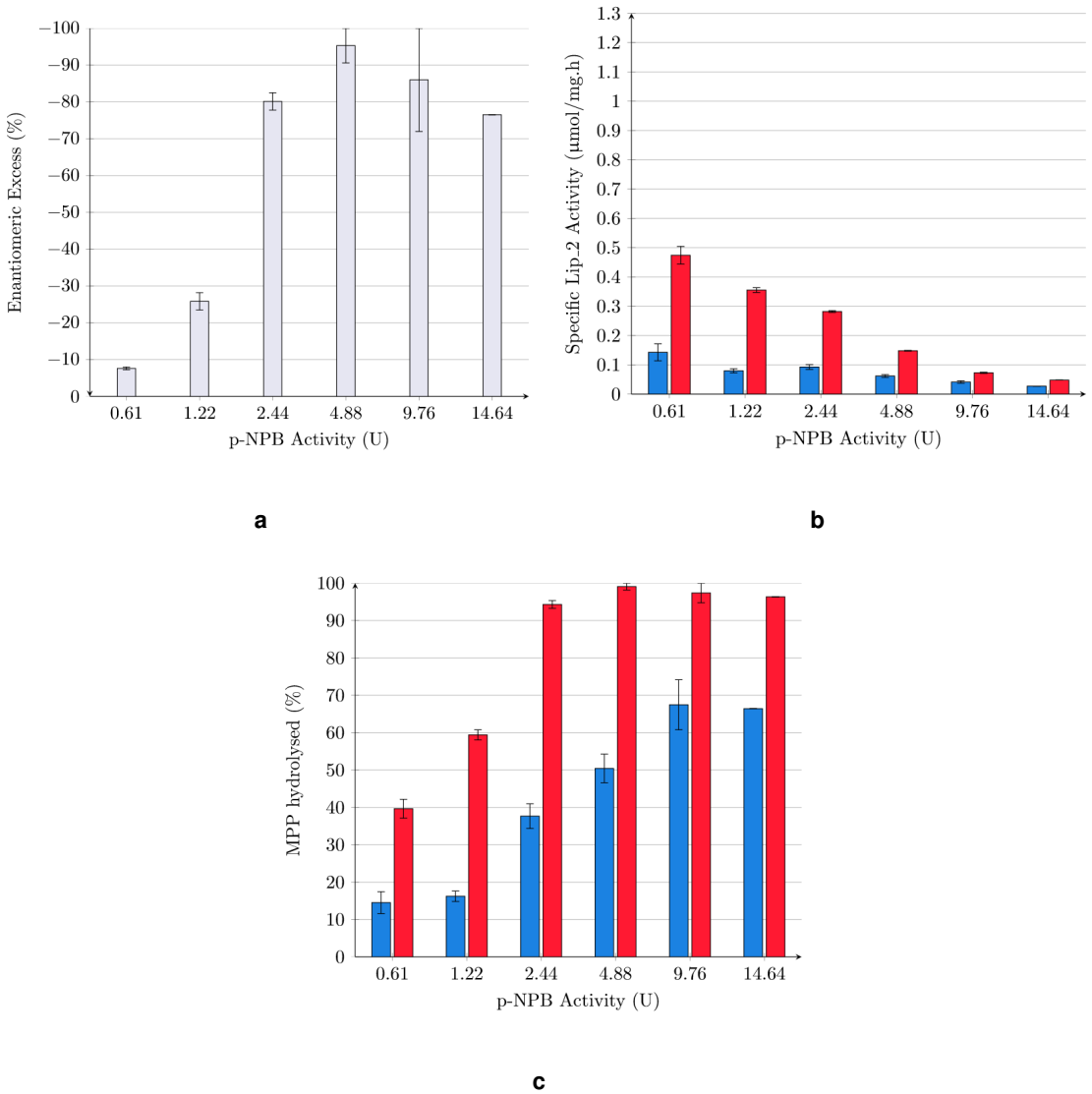


Figure 3.6: Effect of the variation of Lip_2 concentration (quantified through p-NPB activity) on: a the enantiomeric excess of MPP; b specific activity of Lip_2 on the *S* (blue) and *R* (red) enantiomers of MPP; and c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed during the reaction. The initial MPP concentration was 40 mM and the reaction time was 3 h.

The increase of enzyme concentration also led to a decrease of specific activity on both enantiomers as expected (Figure 3.1b). Since the starting substrate concentration was constant the substrate-enzyme ratio decreased with an increase in enzyme concentration.

Lip_2 concentration corresponding to 1.22 U *p*-NPB was chosen for the following assays because at this concentration the enzyme is the limiting factor and to use a smaller amount of cells than the concentration corresponding to 2.44 U *p*-NPB.

3.1.3.B Effect of substrate concentration on Lip_2 activity

With a starting MPP concentration of 20 mM an ee of 55.4 % was achieved (Figure 3.7a). Lip_2 activity on both enantiomers increases slightly until 60 mM of MPP (Figure 3.7b). From 60 to 80 mM of MPP concentration there is a large jump in Lip_2 activity: 8.2 and 2.0 times increase on the activity on the *S*- and *R*-enantiomers of MPP, respectively (Figure 3.7b). Since this assay is done using whole cells as a wet paste this increase can be explained by mass transfer limitations; the quantity of available MPP inside the cells is likely to be much higher when the MPP concentration in the reaction medium reaches a certain value due to highly increased diffusion of MPP into the cells.

60 mM was chosen as the starting substrate concentration for the following assays due to the hydrolysis reaction having the highest selectivity and a low conversion of *(S)*-MPP (Figure 3.7c).

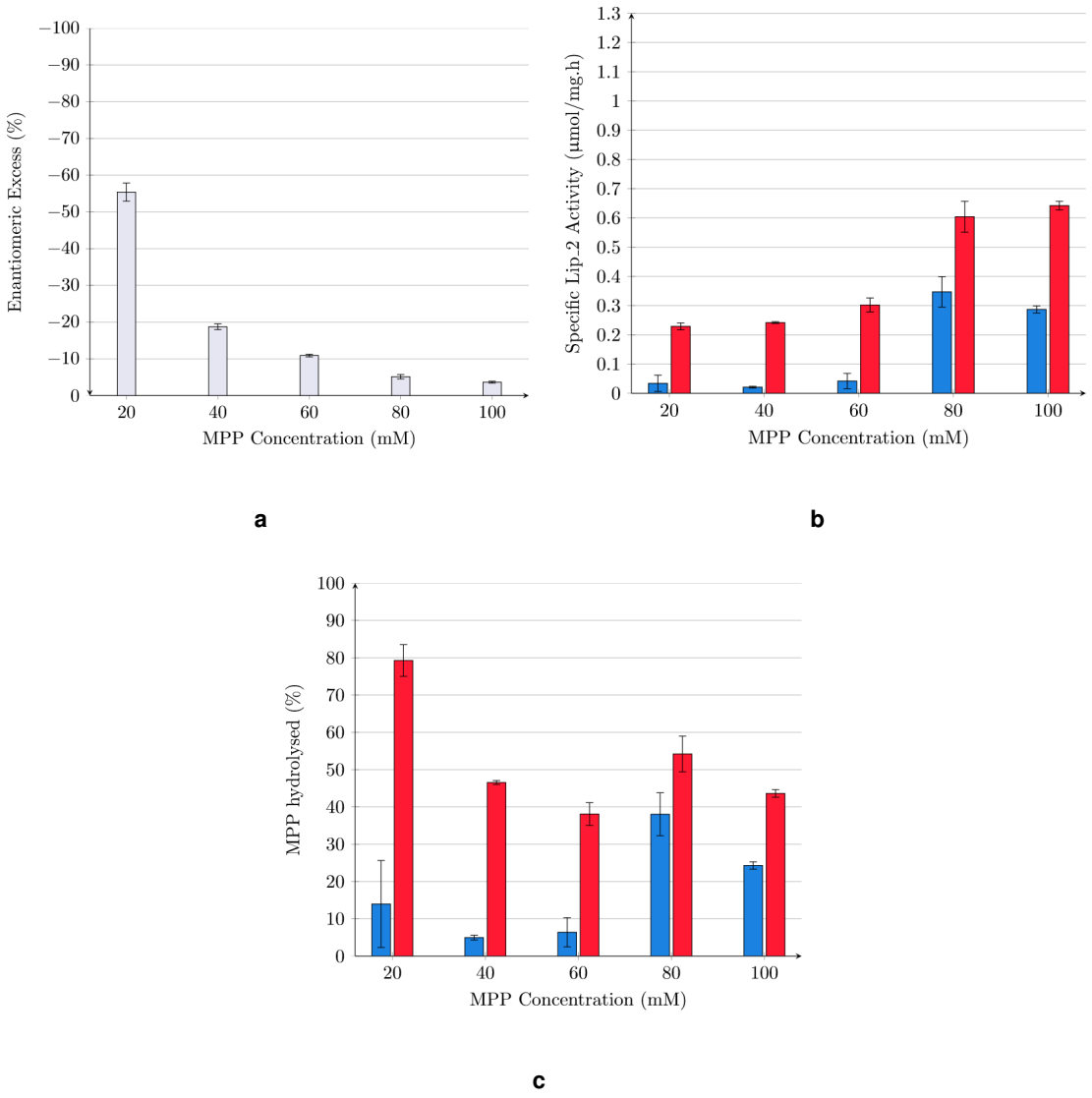


Figure 3.7: Effect of the variation of methyl 2-phenylpropanoate concentration on: a the enantiomeric excess of MPP; b specific activity of Lip_2 on the *S* (blue) and *R* (red) enantiomers of MPP; and c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed during the reaction. The enzyme concentration added corresponded to 1.22 U (*p*-NPB) and the reaction time was 3 h.

3.1.3.C Co-solvent effect on Lip_2 activity

Methanol had a negative effect on Lip_2 enantioselectivity, having reduced Lip_2 activity on the *R*-enantiomer and increasing it on the *S*-enantiomer up to 20 % (v/v) concentration. At 30 % methanol concentration, Lip_2 activity on both the *S*- and *R*-enantiomers of MPP was equal and Lip_2 activity on *(R)*-MPP was 28.3 % of the activity of Lip_2 without methanol present in the reaction system. The presence of DMSO increased Lip_2 activity on both enantiomers and had a diminishing effect with the increase in concentration; 6.4 to 3.6 increase of Lip_2 activity on the *S*-enantiomer from 10 to 30 % DMSO

concentration and 1.63 to 1.35 increase of Lip_2 activity on the *R*-enantiomer on the same DMSO concentration range (Figure 3.8b).

The following assays were done using a 20% DMSO concentration because of the increased Lip_2 activity and balance between conversion and ee (Figures 3.8a and c).

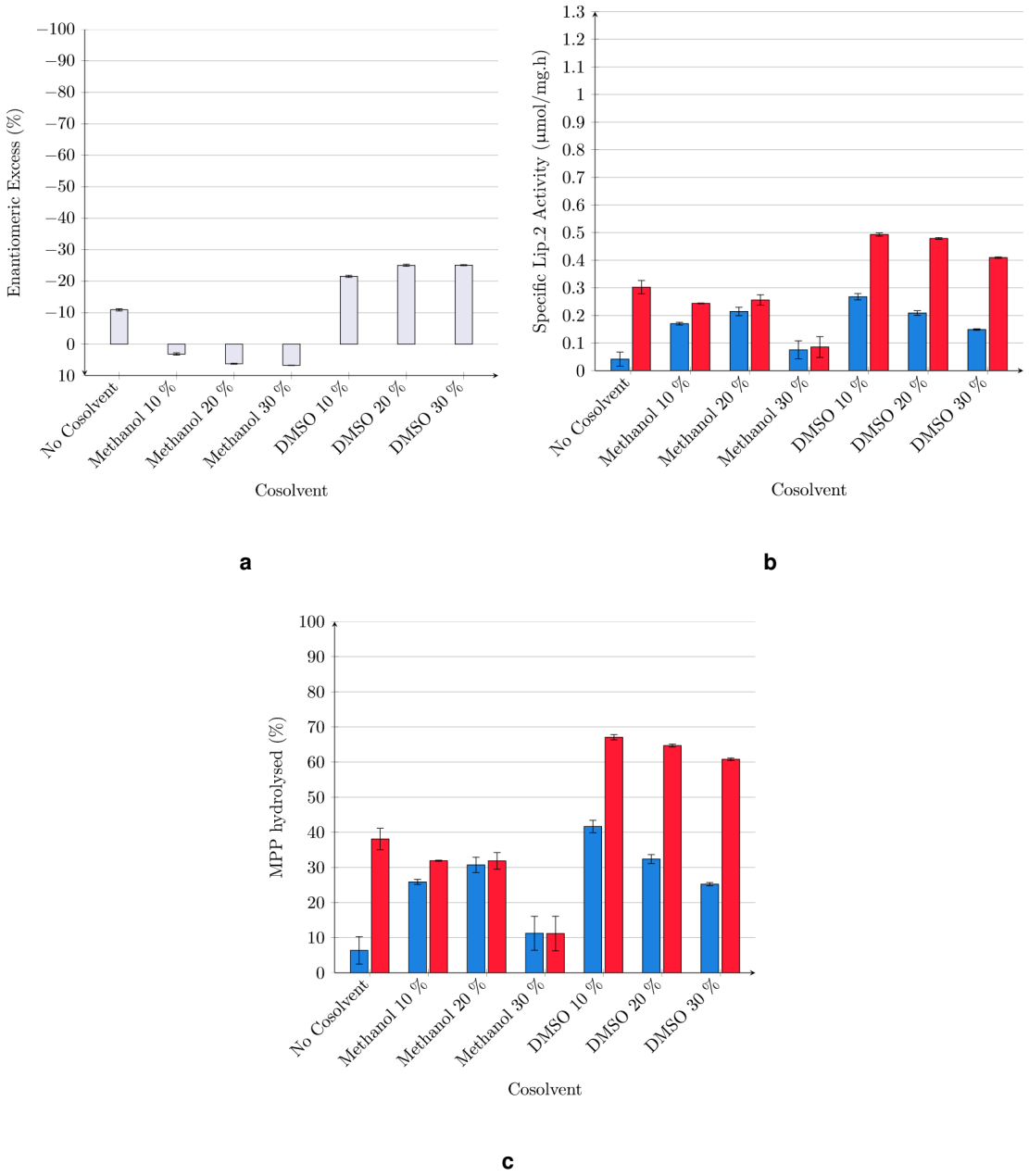


Figure 3.8: Effect of the presence of methanol and DMSO in the reaction media at 10, 20 and 30 %(v/v) on: a the enantiomeric excess of MPP; b specific activity of Lip_2 on the *S* (blue) and *R* (red) enantiomers of MPP; c and percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed during the reaction. The enzyme concentration added corresponded to 1.22 U (*p*-NPB), the initial MPP concentration was 60 mM the reaction time was 3 h.

3.1.3.D Bioreaction run

Both the Lip_2 activity and selectivity on MPP gradually decreased over time: from 1.43 and 1.16 μmol/mg.h on the *R* and *S* enantiomer, respectively, after 1 h, to 0.253 and 0.13 μmol/mg.h on the *R* and *S* enantiomer after 6 h; and, 0.0874 and 0.0863 μmol/mg.h on the *R* and *S* enantiomer after 24 h (Figure 3.9b). The increase in the concentration of *S*-MPP at 3 h is due to an analytical error.

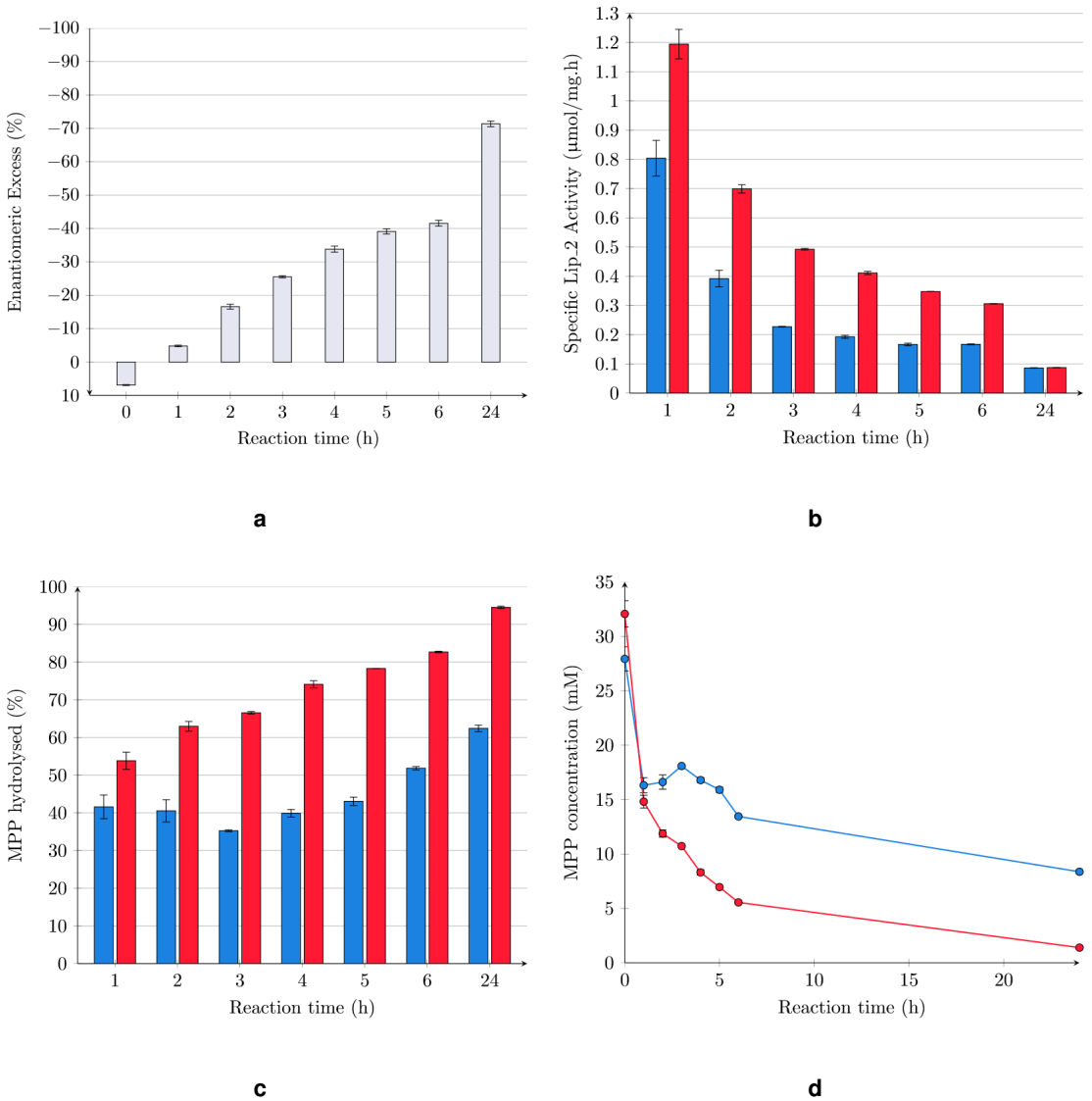


Figure 3.9: Hydrolysis of MPP by Lip_2: a enantiomeric excess of MPP along the reaction progress; b specific activity of Lip_2 on the *S* (blue) and *R* (red) enantiomers of MPP; c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed; and d concentration of the *S* (blue) and *R* (red) enantiomers of MPP over the course of the reaction. The enzyme concentration added corresponded to 1.22 U (*p*-NPB), the initial concentration of MPP was 60 mM and DMSO was used as a co-solvent at 20 %(v/v).

It was not possible to produce enantiomerically pure *(R)*-methyl 2-phenylpropanoate even after 24 h

of reaction. The maximum ee achieved was 71.4 % (Figures 3.9a and d). Due to the reduced selectivity 62.4 % of *(S)*-MPP is hydrolysed which leads to a yield of 37.6 %.

3.1.3.E Scale-up

An increase in the reaction volume led to a decrease in selectivity. This was due to a 18.9 % decrease of Lip_2 activity on the *R* enantiomer (Figure 3.10b) which led to reduced ee and *(R)*-MPP conversion (Figures 3.10a and c). This was due to mass transfer limitations since the radius of the average droplet of MPP increased with the reaction volume, which decreased the specific area of the droplets, which led to worse mass transfer, which reduced the available MPP in contact with the enzyme, which naturally reduced the selectivity, as explained in section 3.1.2.E [83].

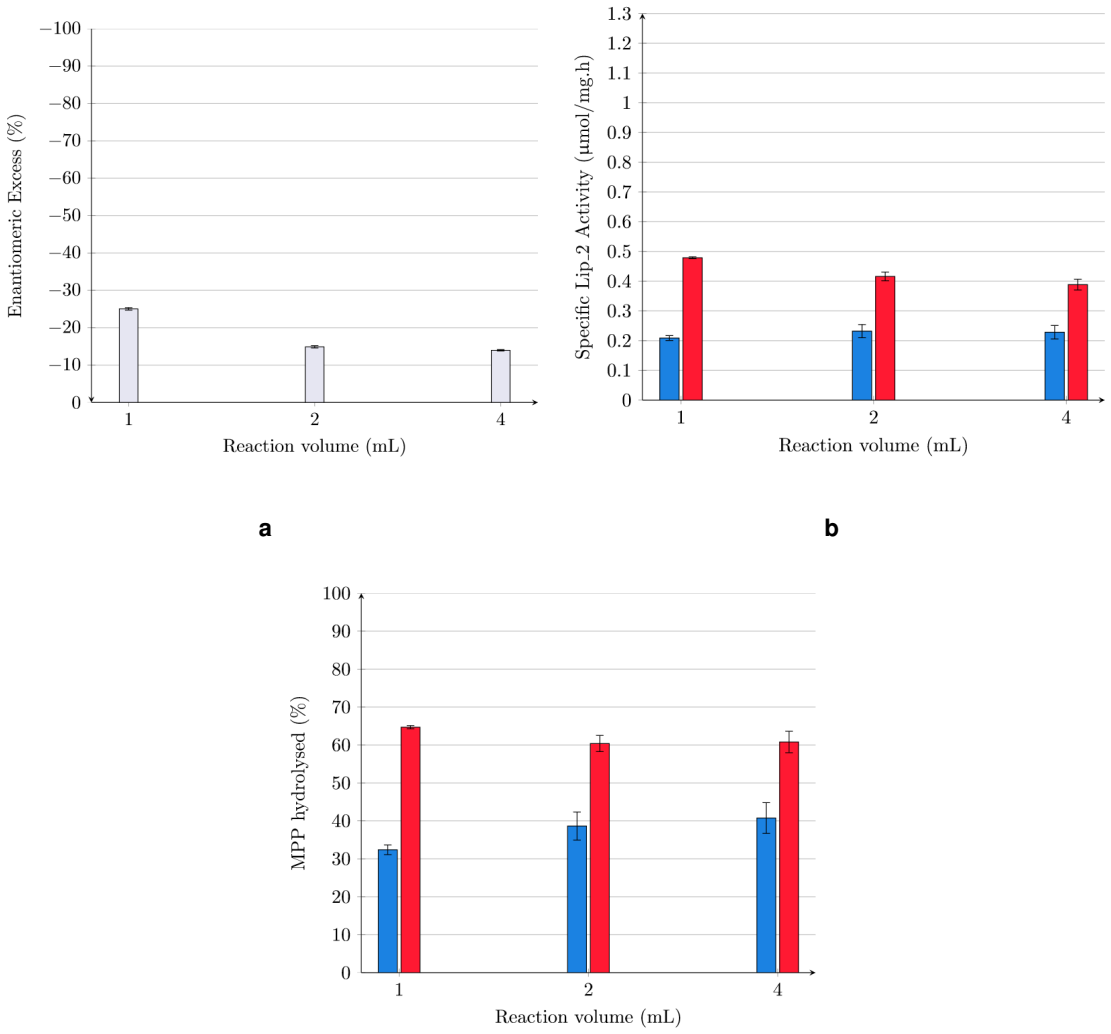


Figure 3.10: Effect of doubling and quadrupling the reaction volume on: a the enantiomeric excess of MPP; b specific activity of Lip_2 on the *S* (blue) and *R* (red) enantiomers of MPP; and c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed during the reaction. The enzyme concentration added corresponded to 1.22 U (*p*-NPB), the initial concentration of MPP was 60 mM, DMSO was used as a co-solvent at 20 %v and the reaction time was 3 h.

3.2 Production of Lip_2 overexpressing *E. coli* cells

The Lip_2 expressing cells were grown in-house because they were found to quickly lose activity during the transport from Germany [84]. The induction medium composition was also optimized. The starting induction medium (Table 3.2) was developed in Heinrich Heine University of Düsseldorf by the group of Professor Karl-Erich Jaeger. The pre-inoculum was grown in LB medium supplemented with 0.5 % glucose and ampicillin 100 μg/mL.

Table 3.2: Growth medium for *E. coli* BL21 (DE3) pET22b_Lip_2.

Component	Concentration
Na_2HPO_4	3.1 g/L
KH_2PO_4	1.7 g/L
Tryptone	20 g/L
Yeast extract	5 g/L
NaCl	5 g/L
Ampicillin	100 μg/mL
Glucose	0.05 %m
Glycerol	0.6 %v
Lactose	0.2 %m

3.2.1 Growth curve

The growth curves of *E. coli* BL21 (DE3) pET22b_Lip_2 were obtained by measuring the OD of the cell suspension at 580 nm.

The three stages of microbial growth in a batch mode of operation can clearly be seen on the growth curves (Figure 3.11). A lag phase from the start until 7 h on the LB medium and not present on the induction medium, which is expected since it is inoculated with active cells instead of cells stored at 4°C. An exponential phase was observed from 7 h until 14 h on the LB medium and from the start until 4 h on the induction medium. A stationary phase was observed from the end of the exponential phase until the end of the curve on both culture media.

The maximum specific growth rate (μ_{max}) (Table 3.3) can be calculated by the slope of the OD line in the exponential phase in the semi-logarithmic growth curve plots (Figures 3.11c and d).

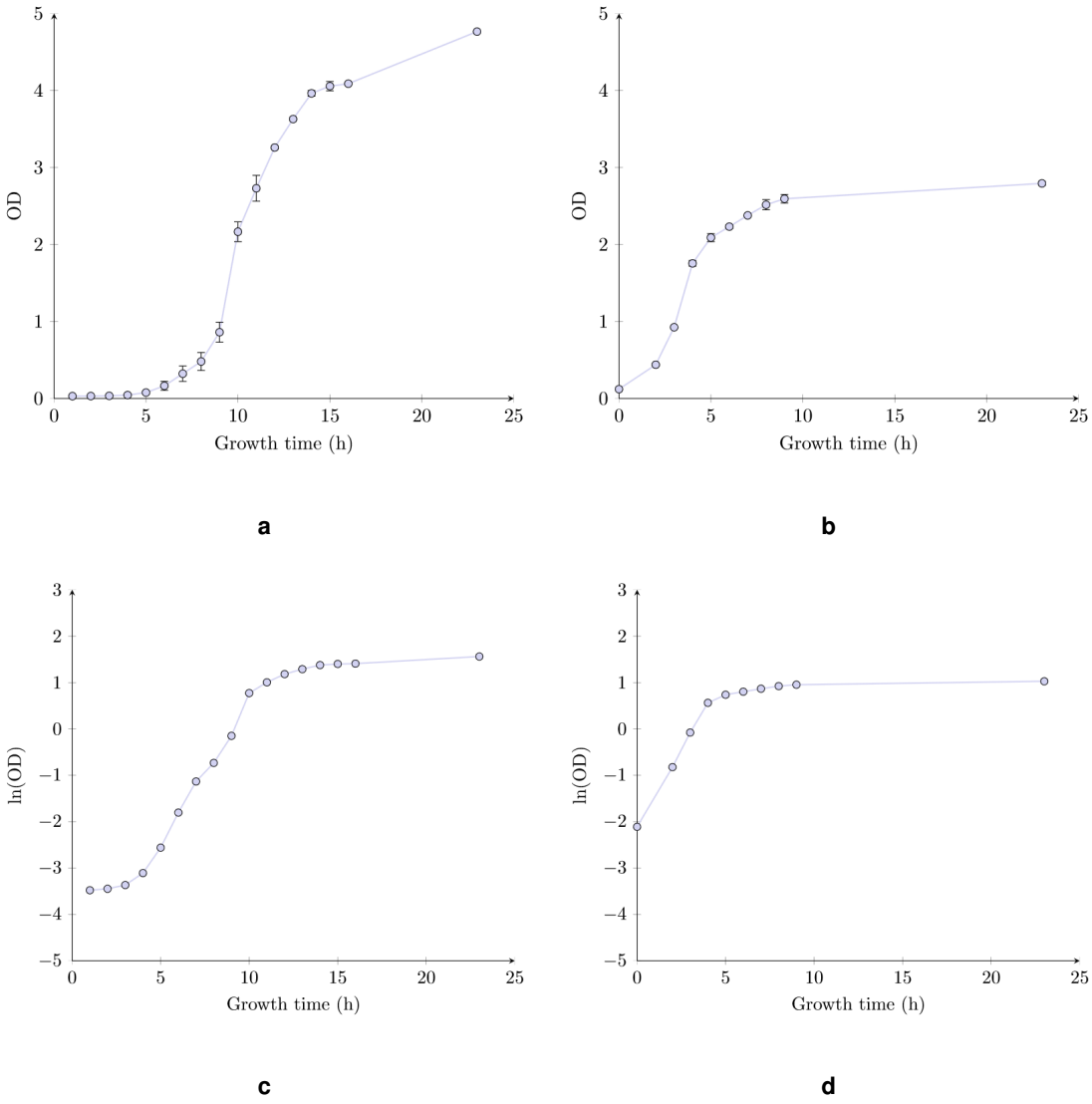


Figure 3.11: Growth curves for *E. coli* BL21 (DE3) pET22b_Lip_2 in LB medium a and in standard induction medium b at 30 °C and 160 rpm, and with the corresponding semi-logarithmic cd plots.

Table 3.3: Values of μ_{max} and $t_{½}$ for *E. coli* BL21 (DE3) pET22b_Lip_2 in LB and the standard induction media at 30°C and 160 rpm orbital shaking.

	μ_{max} (h^{-1})	$t_{½}$ (min)
LB	0.668	62.2
Induction	0.672	61.9

The obtained values for the doubling time were much higher than the minimum possible doubling time for *E. coli* in laboratory conditions 30 min at 30 °C according to the literature [85], which is to be expected due to Lip_2 expression.

3.2.2 Optimization of the growth medium composition

3.2.2.A Necessity of glycerol and lactose

The influence of supplementing the growth medium with glycerol and lactose was assessed by growing the cells without each of these carbon sources (Table 3.4). Removing either of the carbon sources from the culture media resulted in reduced esterase activity.

Table 3.4: Effect of removing glycerol and lactose from the expression medium on the specific esterase activity of the cells measured in U (p-NPB)/g.

Growth medium	Esterase activity U (*p*-NPB)/g
Induction medium	223.2
Induction medium without glycerol	191.1
Induction medium without lactose	148.4

3.2.2.B Effect of the supplementary carbon sources

The effect of the concentration of glucose, glycerol and lactose on cell growth and enzymatic activity was tested. In order to assess the growth of multiple growth conditions with high-throughput, a 14 well plate OxoDish® OD24 with SDR SensorDish® Reader from PreSens Precision Sensing GmbH was used. This system consists of a 24 deep-well microplate with an oxygen sensor and a transparent bottom on each well and a 24 channel reader that reads non-invasively through the transparent bottom [86, 87]. It allows for online measurement of the dissolved oxygen (DO) in the cell growth suspension, and its depletion rate is related to the growth rate. Due to the small amount of medium, sterile sealing tapes were used to prevent evaporation [88].

In shaked cell cultures oxygen is usually the limiting nutrient. In the oxygen profile, there are two clearly different regions corresponding to the lag and exponential phases (Figure 3.12). During the lag phase, the oxygen consumption is very low, while during the exponential phase the culture will deplete all the available oxygen in the suspension. During the stationary phase (not shown), oxygen consumption will decrease and the DO of the suspension will again increase.

The depletion rate of the DO, measured by the slope of the DO concentration is related to the maximum specific growth rate of the culture (Table 3.5). In the oxygen profile, there is clear indication of diauxic growth (Figure 3.12). Oxygen profiles for all tested growth conditions are available at Appendix C.

Multiple combinations of supplementary carbons were tested for the effect on the growth rate and on the enzymatic activity (Table 3.6). An increase in glucose concentration has clear inhibitory effect on the

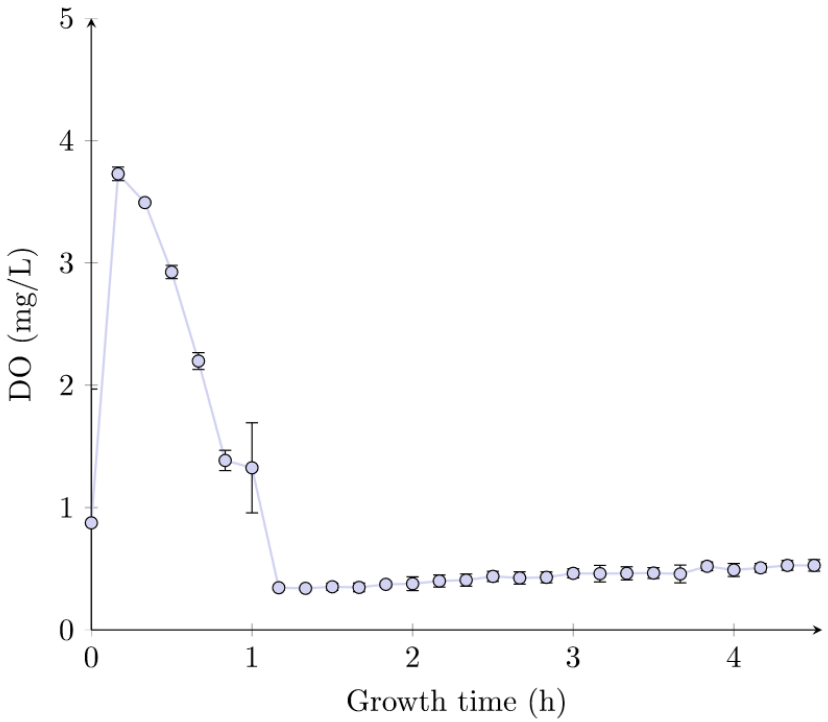


Figure 3.12: Oxygen profile measured with the SDR SensorDish® Reader in the well of an a OxoDish® OD24 micro titer plate for *E. coli* BL21 (DE3) pET22b_Lip_2 using the standard medium supplemented with 0.05 %m glucose, 0.6 %v glycerol and 0.5 %m lactose, 30 °C and 160 rpm.

Table 3.5: Oxygen consumption rate for both exponential phases of the diauxic growth of *E. coli* BL21 (DE3) pET22b_Lip_2 using the standard medium supplemented with 0.05 %(m/v) glucose, 0.6 %(v/v) glycerol and 0.5 %(m/v) lactose at 30 °C and 160 rpm orbital shaking.

	Oxygen depletion rate (mg/(L.h))	Start (h)	End (h)
First exponential phase	3.59	0.167	0.834
Second exponential phase	5.88	1.000	1.167

p-NPB activity of the cells. An increase in glycerol concentration (after 0.1 %) has a positive effect on the *p*-NPB activity of the cells having a maximum at 0.6 %. However the *p*-NPB activity of the cells is highest with 0.05 % glycerol which indicates that when the cells trigger the necessary mechanisms to metabolize glycerol Lip_2 expression is reduced. An increase in lactose concentration increases the *p*-NPB activity of the cells which is to be expected because the strain has the gene that codifies the enzyme Lip_2 built into the *Lac* operon. However the data only shows this trend for a glucose concentration in the medium of 0.05 %. For higher glucose concentrations the lower lactose concentration shows higher *p*-NPB activity. On the laboratorial scale the growth rate of the cells is irrelevant since the cells can only be grown overnight, however on an industrial scale it must be taken into consideration and further studies must be done on it and, most importantly, on the variation of enzymatic activity on *p*-NPB hydrolysis

during the different stages of cellular growth.

Table 3.6: Effect of changing the growth medium concentration of glucose (Glu), glycerol (Gly) and lactose (Lac) in the esterase activity of cells (p-NPB) measured in U (p-NPB)/g and on cell growth (Growth) measured consumption of oxygen - mg/L.h. Green represents the maximum value while red represents the minimum value. All tests were done at 30 °C and 160 rpm. The media supplemented with (i) 0.05 % glucose, 0.6 % glycerol, 0.2 % lactose and (ii) 0.05 % glucose, 0.6 % glycerol, 0.5 % lactose present diauxic growth with oxygen consumption rates of 7.89 and 5.88 mg/(L.h), respectively.

Glu	Gly	Lac	p-NPB	Growth
0.05	0.6	0.2	186	3.54
0.5	0.6	0.2	50.6	4.14
0.75	0.6	0.2	49.9	4.17
1	0.6	0.2	25.0	4.21
0.05	0.6	0.5	225	3.58
0.5	0.6	0.5	28.1	3.85
0.75	0.6	0.5	20.5	4.06
1	0.6	0.5	15.6	4.11
0.5	0.05	0.2	54.5	4.14
0.5	0.1	0.2	18.8	4.17
0.5	0.25	0.2	29.2	4.08
0.5	0.8	0.2	41.7	3.99

4

Conclusion

Contents

4.1 Conclusions

Lip_1 was found to selectively hydrolyse the *(S)*-enantiomer of MPP. Under the optimal conditions enantiopure *(R)*-MPP was achieved after one hour of reaction with a total specific Lip_1 activity of 272.7 μmol MPP/mg.h and a yield of 45.4 %. Lip_2 was found to selectively hydrolyse the *(R)*-enantiomer of MPP. Even under the optimal conditions it was impossible to obtain enantiopure *(S)*-MPP after twenty-four hours of reaction. The average total specific Lip_2 activity was 0.174 μmol MPP/mg.h, the final ee of MPP was -71.4 % with a yield of 37.6 %.

Lip_1 clearly shows a better catalytic potential than Lip_2 due to having a specific activity 3 orders of magnitude above Lip_2, however the reaction yields of both enzyme catalysed reactions are low. Due to this, this system can only be applied to manufacture compounds with a very high added value from this biotransformation.

In both reaction systems the increase in scale resulted in reduced enzyme specificity due to mass transfer limitations, further indicating the difficulty of commercial applications.

Towards the end of the INMARE project it was found that while low enzymatic promiscuity does not imply high enantioselectivity, there is a clear negative correlation between high enzymatic promiscuity and enantioselectivity [89]. The obtained low selectivity is then to be expected due to the facts that the enzymes were selected for their promiscuity and the model substrate (MPP) was chosen for the difficulty of its chiral synthesis and resolution due to low steric hindrance effects.

4.2 Future Work

Due to time constraints caused by the COVID-19 pandemic and problems with the Lip_2 reaction system, the proposed reaction systems were not completely studied. Several tests were supposed to have been conducted, including the following:

- The partition of MPP in the water/hexane system, in order to more accurately calculate the hydrolysis rate;
- The evaluation of Lip_2 concentration along the cellular growth, in order to identify the optimal point in time to stop biomass production and harvest the cells, which is imperative in an industrial setting;
- The effect of the cellular disruption method. Cellular disruption would have alleviated the mass transfer limitations present in this work. Lip_1 cells were lyophilized and Lip_2 cells were used whole; lyophilization is not viable due its cost when used at an industrial scale. High pressure homogenization and chemical disruption through the addition of an easily separable organic solvent (such as chloroform) are the ideal candidates;

- The effect of enzyme immobilization. Enzyme immobilization can greatly improve an enzyme's commercial viability due to increased activity, thermal stability and activity retention. Immobilized enzymes also allow for vastly easier separation.

Since cells are a complex system, in order to confirm that the hydrolytic activity is, in fact, due to Lip_1 or Lip_2, and not due to another enzyme it is necessary to purify and assess the activity of pure Lip_1 and Lip_2. Due to enzyme purification being a costly procedure it is unlikely that pure enzymes would make a commercially viable catalyst. However that is possible and the assessment of immobilized pure Lip_1 and Lip_2 should also be considered.

Due to a lack of equipment it was impossible to analyse the chirality of the reaction product 2-phenylpropionic acid. This molecule is a profen and its chiral synthesis can be important. The assessment of the chiral configuration of the reaction product of this reaction system is therefore an important item to finalize this study.

The INMARE goal of finding enantioseletive promiscuous enzymes could not be reached since these properties were found to be opposite of one another. Should Lip_1 and Lip_2 ultimately fail to perform adequately from a commercial standpoint then bioprospecting for new enzymes is the only remaining option.

Bibliography

[1] L. Pauling, “Nature of forces between large molecules of biological interest.” *Nature*, vol. 161, no. 4097, p. 707, 1948.

[2] J. C. Aledo, C. Lobo, and A. E. del Valle, “Energy diagrams for enzyme-catalyzed reactions: Concepts and misconcepts,” *Biochemistry and Molecular Biology Education*, vol. 31, no. 4, pp. 234–236, 2003.

[3] P. L. B. Trevor Palmer, *Enzymes: Biochemistry, Biotechnology, Clinical Chemistry*, 2nd ed. Woodhead Publishing, 2007.

[4] R. Hill, *The chemistry of life: eight lectures on the history of biochemistry*. CUP Archive, 1970.

[5] A. Payen and J.-F. Persoz, “Mémoire sur la diastase, les principaux produits de ses réactions, et leurs applications aux arts industriels,” *Ann. chim. phys*, vol. 53, pp. 73–92, 1833.

[6] J. Basslinger, *Pepsin, seine physiologischen Erscheinungen und therapeutischen Wirkungen gegen Verdauungsschwäche*, 1858.

[7] W. Kirchner, *Beiträge zur Kenntniss der Kuhmilch und ihrer Bestandtheile nach dem gegenwärtigen Standpunkte wissenschaftlicher Forschung*. Schönfeld, 1877.

[8] E. Buchner and R. Rapp, “Alkoholische gährung ohne hefezellen,” *Berichte der deutschen chemischen Gesellschaft*, vol. 30, no. 3, pp. 2668–2678, 1897.

[9] J. B. Sumner, “The isolation and crystallization of the enzyme urease preliminary paper,” *Journal of Biological Chemistry*, vol. 69, no. 2, pp. 435–441, 1926.

[10] E. Nomenclature, “Recommendations 1964 of the international union of biochemistry,” *Amsterdam: Elsevier Publication Comp*, 1965.

[11] IUPAC-IUBMB. (2020) IUPAC-IUBMB joint commission on biochemical nomenclature (jcbn). Acessed 29th June 2020. [Online]. Available: https://www.qmul.ac.uk/sbcs/iubmb/nomenclature/

[12] M. M. C. David L. Nelson, *Lehninger Principles of Biochemistry, 5th Edition*, 5th ed. W. H. Freeman, 2008.

[13] Department of Bioinformatics and Biochemistry, Institute for Biochemistry and Biotechnology, Technishche Universität Braunschweig. (2020) Brenda (the comprehensive enzyme information system). Acessed 27th November 2020. [Online]. Available: https://www.brenda-enzymes.org/ecexplorer.php?browser=1&f[nodes]=&f[action]=open&f[change]=358

[14] D.-T. Tran, C.-L. Chen, and J.-S. Chang, "Immobilization of *Burkholderia sp.* lipase on a ferric silica nanocomposite for biodiesel production," *Journal of biotechnology*, vol. 158, no. 3, pp. 112–119, 2012.

[15] J. S. Lupoi and E. A. Smith, "Evaluation of nanoparticle-immobilized cellulase for improved ethanol yield in simultaneous saccharification and fermentation reactions," *Biotechnology and bioengineering*, vol. 108, no. 12, pp. 2835–2843, 2011.

[16] D. Pollard, M. Truppo, J. Pollard, C.-y. Chen, and J. Moore, "Effective synthesis of *(S)*-3, 5-bistrifluoromethylphenyl ethanol by asymmetric enzymatic reduction," *Tetrahedron: Asymmetry*, vol. 17, no. 4, pp. 554–559, 2006.

[17] W. A. Greenberg, A. Varvak, S. R. Hanson, K. Wong, H. Huang, P. Chen, and M. J. Burk, "Development of an efficient, scalable, aldolase-catalyzed process for enantioselective synthesis of statin intermediates," *Proceedings of the National Academy of Sciences*, vol. 101, no. 16, pp. 5788–5793, 2004.

[18] Y. Asano, T. Yasuda, Y. Tani, and H. Yamada, "A new enzymatic method of acrylamide production," *Agricultural and biological chemistry*, vol. 46, no. 5, pp. 1183–1189, 1982.

[19] Y. Takasaki, "Studies on sugar-isomerizing enzyme: Production and utilization of glucose isomerase from *Streptomyces sp.*" *Agricultural and Biological Chemistry*, vol. 30, no. 12, pp. 1247–1253, 1966.

[20] R. A. Sheldon and P. C. Pereira, "Biocatalysis engineering: the big picture," *Chemical Society Reviews*, vol. 46, no. 10, pp. 2678–2691, 2017.

[21] J. Uppenberg, M. T. Hansen, S. Patkar, and T. A. Jones, "The sequence, crystal structure determination and refinement of two crystal forms of lipase b from *Candida antarctica*," *Structure*, vol. 2, no. 4, pp. 293–308, 1994.

[22] R. Verger, "'Interfacial activation' of lipases: facts and artifacts," *Trends in Biotechnology*, vol. 15, no. 1, pp. 32–38, 1997.

[23] H. Chahinian, L. Nini, E. Boitard, J.-P. Dubès, L.-C. Comeau, and L. Sarda, "Distinction between esterases and lipases: a kinetic study with vinyl esters and tag," *Lipids*, vol. 37, no. 7, pp. 653–662, 2002.

[24] P. Fojan, P. H. Jonson, M. T. Petersen, and S. B. Petersen, "What distinguishes an esterase from a lipase: a novel structural approach," *Biochimie*, vol. 82, no. 11, pp. 1033–1041, 2000.

[25] D. L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S. M. Franken, M. Harel, S. J. Remington, I. Silman, J. Schrag *et al.*, "The α/β hydrolase fold," *Protein Engineering, Design and Selection*, vol. 5, no. 3, pp. 197–211, 1992.

[26] P. D. Carr and D. L. Ollis, "α/βhydrolase fold: an update," *Protein and peptide letters*, vol. 16, no. 10, pp. 1137–1148, 2009.

[27] A. Rauwerdink and R. J. Kazlauskas, "How the same core catalytic machinery catalyzes 17 different reactions: the serine-histidine-aspartate catalytic triad of α/β-hydrolase fold enzymes," *ACS catalysis*, vol. 5, no. 10, pp. 6153–6176, 2015.

[28] L. Brady, A. M. Brzozowski, Z. S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J. P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov *et al.*, "A serine protease triad forms the catalytic centre of a triacylglycerol lipase," *Nature*, vol. 343, no. 6260, pp. 767–770, 1990.

[29] J. D. Schrag and M. Cygler, "[4] lipases and α/β hydrolase fold," in *Methods in enzymology*. Elsevier, 1997, vol. 284, pp. 85–107.

[30] L. Casas-Godoy, S. Duquesne, F. Bordes, G. Sandoval, and A. Marty, "Lipases: an overview," in *Lipases and phospholipases*. Springer, 2012, pp. 3–30.

[31] S. Hari Krishna and N. Karanth, "Lipases and lipase-catalyzed esterification reactions in nonaqueous media," *Catalysis Reviews*, vol. 44, no. 4, pp. 499–591, 2002.

[32] P. Chandra, R. Singh, P. K. Arora *et al.*, "Microbial lipases and their industrial applications: a comprehensive review," *Microbial Cell Factories*, vol. 19, no. 1, pp. 1–42, 2020.

[33] D. Titus, E. J. J. Samuel, and S. M. Roopan, "Importance of food science and technology-way to future," in *Bioorganic Phase in Natural Food: An Overview*. Springer, 2018, pp. 11–23.

[34] A. Idris and A. Bukhari, "Immobilized *Candida antarctica* lipase b: Hydration, stripping off and application in ring opening polyester synthesis," *Biotechnology advances*, vol. 30, no. 3, pp. 550–563, 2012.

[35] S. Cherif, S. Mnif, F. Hadrich, S. Abdelkafi, and S. Sayadi, “A newly high alkaline lipase: an ideal choice for application in detergent formulations,” *Lipids in health and disease*, vol. 10, no. 1, pp. 1–8, 2011.

[36] C. H. Okino-Delgado, D. Z. d. Prado, R. Facanali, M. M. O. Marques, A. S. Nascimento, C. J. d. C. Fernandes, W. F. Zambuzzi, and L. F. Fleuri, “Bioremediation of cooking oil waste using lipases from wastes,” *PLoS One*, vol. 12, no. 10, 2017.

[37] C. C. Akoh, S.-W. Chang, G.-C. Lee, and J.-F. Shaw, “Enzymatic approach to biodiesel production,” *Journal of agricultural and food chemistry*, vol. 55, no. 22, pp. 8995–9005, 2007.

[38] R. Irimescu, K. Furihata, K. Hata, and T. Yamane, “Process for the production of glycerides with lipases,” Jun. 14 2005, US Patent 6,905,850.

[39] M. M. R. D. Fonseca, N. M. F. Osório, and S. F. Dias, “Continuous process of transesterification of fats catalyzed by lipases obtains fats useful in the food industry with rheological characteristics different to those of original mixture,” Jun. 21 2001, PT Patent 102,638.

[40] F. V. Kosikowski and R. C. Jolly, “Flavor development by microbial lipases in pasteurized milk blue cheese,” Aug. 3 1976, US Patent 3,973,042.

[41] M. W. Rey, E. J. Golightly, and T. Spendler, “Methods for using lipases in baking,” May 4 2004, US Patent 6,730,346.

[42] P. Julien, M.-P. Lejeune-Luquet, and E. Schubert, “Bread improver,” Oct. 19 2005, EU patent 1586240.

[43] S.-W. Tsai, “Enzymatic resolution of an alpha-substituted carboxylic acid or an ester thereof by carica papaya lipase,” Jan. 5 2006, US Patent App. 11/168,490.

[44] P. Cesti and P. Piccardi, “Process for the biotechnological preparation of optically active alpha-arylalkanoic acids,” Aug. 9 1988, US Patent 4,762,793.

[45] A. Day, H. Dillon, G. Brooks, and S. Franklin, “Direct chemical modification of microbial biomass and microbial oils,” Jun. 30 2009, Patent JP 2016116535.

[46] W. Aehle, G. Gerritse, and H. B. Lenting, “Lipases with improved surfactant resistance,” Jan. 25 2000, US Patent 6,017,866.

[47] J. Haerer, P. Jeschke, and C. Nitsch, “Use of lipases in low-alkaline mechanical dishwashing agents,” Mar. 06 1997, WO 9708281.

[48] C. Ortiz, M. L. Ferreira, O. Barbosa, J. C. dos Santos, R. C. Rodrigues, Á. Berenguer-Murcia, L. E. Briand, and R. Fernandez-Lafuente, "Novozym 435: the "perfect" lipase immobilized biocatalyst?" *Catalysis Science & Technology*, vol. 9, no. 10, pp. 2380–2420, 2019.

[49] D. R. Klein, *Organic Chemistry*, 1st ed. Wiley, 2011.

[50] C. C. C. R. Carvalho and M. M. R. da Fonseca, "Carvone: Why and how should one bother to produce this terpene," *Food Chemistry*, vol. 95, no. 3, pp. 413–422, 2006.

[51] E. Tokunaga, T. Yamamoto, E. Ito, and N. Shibata, "Understanding the thalidomide chirality in biological processes by the self-disproportionation of enantiomers," *Scientific reports*, vol. 8, no. 1, pp. 1–7, 2018.

[52] G. Blaschke, H. Kraft, K. Fickentscher, and F. Köhler, "Chromatographic separation of racemic thalidomide and teratogenic activity of its enantiomers (author's transl)," *Arzneimittel-forschung*, vol. 29, no. 10, p. 1640, 1979.

[53] National Archives and Records Administration. (2020) Act of october 10, 1962 (drug amendments act of 1962), public law 87-781, 76 stat 780, which amended the federal food, drug, and cosmetic act to assure the safety, effectiveness, and reliability of drugs, authorize standardiztion of drug names, and clarify and strengthen existing inspection authority. Acessed 10th December 2020. [Online]. Available: https://catalog.archives.gov/id/299878

[54] EUR-Lex. (2020) Council directive 65/65/eec of 26 january 1965 on the approximation of provisions laid down by law, regulation or administrative action relating to proprietary medicinal products. Acessed 10th December 2020. [Online]. Available: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31965L0065:EN:HTML

[55] European Medicines Agency. (2020) Investigation of chiral active substances (human). Acessed 10th December 2020. [Online]. Available: https://www.ema.europa.eu/en/investigation-chiral-active-substances-human

[56] Food and Drug Administration. (2020) Development of new stereoisomeric drugs. Acessed 10th December 2020. [Online]. Available: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/development-new-stereoisomeric-drugs

[57] L. Yu, S. Wang, and S. Zeng, "Chiral mobile phase additives in HPLC enantioseparations," in *Chiral Separations*. Springer, 2013, pp. 221–231.

[58] G. Gübitz and M. G. Schmid, "Chiral separation by chromatographic and electromigration techniques. a review," *Biopharmaceutics & drug disposition*, vol. 22, no. 7-8, pp. 291–336, 2001.

[59] L. C. Hsu, H. Kim, X. Yang, and D. Ross, “Large scale chiral chromatography for the separation of an enantiomer to accelerate drug development,” *Chirality*, vol. 23, no. 4, pp. 361–366, 2011.

[60] T. Toyo’oka, “Recent progress in liquid chromatographic enantioseparation based upon diastereomer formation with fluorescent chiral derivatization reagents,” *Biomedical Chromatography*, vol. 10, no. 6, pp. 265–277, 1996.

[61] R. A. Rajewski, D. G. Kosednar, T. A. Matches, O. S. Wong, K. Burchett, and K. Thakker, “Stereospecific analysis of a novel protein kinase c inhibitor,” *Journal of pharmaceutical and biomedical analysis*, vol. 13, no. 3, pp. 247–253, 1995.

[62] H. Frank, G. J. Nicholson, and E. Bayer, “Rapid gas chromatographic separation of amino acid enantiomers with a novel chiral stationary phase,” *Journal of Chromatographic Science*, vol. 15, no. 5, pp. 174–176, 1977.

[63] H. Frank, G. J. Nicholson, and E. Bayer, “Enantiomer labelling, a method for the quantitative analysis of amino acids,” *Journal of Chromatography A*, vol. 167, pp. 187–196, 1978.

[64] J. W. Westley and B. Halpern, “Use of (-)-menthyl chloroformate in the optical analysis of asymmetric amino and hydroxyl compounds by gas chromatography,” *The Journal of Organic Chemistry*, vol. 33, no. 10, pp. 3978–3980, 1968.

[65] N. Singh, F. Pasutto, R. Coutts, and F. Jamali, “Gas chromatographic separation of optically active anti-inflammatory 2-arylpropionic acids using (+)-or (-)-amphetamine as derivatizing reagent,” *Journal of Chromatography B: Biomedical Sciences and Applications*, vol. 378, pp. 125–135, 1986.

[66] A. Kamal, M. A. Azhar, T. Krishnaji, M. S. Malik, and S. Azeeza, “Approaches based on enzyme mediated kinetic to dynamic kinetic resolutions: A versatile route for chiral intermediates,” *Coordination Chemistry Reviews*, vol. 252, no. 5-7, pp. 569–592, 2008.

[67] R. S. Ward, “Dynamic kinetic resolution,” *Tetrahedron: Asymmetry*, vol. 6, no. 7, pp. 1475–1490, 1995.

[68] M. Jacobs, M. Eliasson, M. Uhlén, and J.-I. Flock, “Cloning, sequencing and expression of subtilisin carlsberg from *Bacillus licheniformis*,” *Nucleic Acids Research*, vol. 13, no. 24, pp. 8913–8926, 1985.

[69] M. T. Reetz, W. Wiesenhöfer, G. Francio, and W. Leitner, “Continuous flow enzymatic kinetic resolution and enantiomer separation using ionic liquid/supercritical carbon dioxide media,” *Advanced Synthesis & Catalysis*, vol. 345, no. 11, pp. 1221–1228, 2003.

[70] L. Borén, B. Martín-Matute, Y. Xu, A. Córdova, and J.-E. Bäckvall, "(s)-selective kinetic resolution and chemoenzymatic dynamic kinetic resolution of secondary alcohols," *Chemistry–A European Journal*, vol. 12, no. 1, pp. 225–232, 2006.

[71] J.-S. Shin and B.-G. Kim, "Kinetic modeling of ω-transamination for enzymatic kinetic resolution of α-methylbenzylamine," *Biotechnology and bioengineering*, vol. 60, no. 5, pp. 534–540, 1998.

[72] S. J. Taylor, A. G. Sutherland, C. Lee, R. Wisdom, S. Thomas, S. M. Roberts, and C. Evans, "Chemoenzymatic synthesis of (–)-carbovir utilizing a whole cell catalysed resolution of 2-azabicyclo [2.2.1] hept-5-en-3-one," *Journal of the Chemical Society, Chemical Communications*, no. 16, pp. 1120–1121, 1990.

[73] R. E. Gawley and J. Aube, *Principles of Asymmetric Synthesis*, 2nd ed. Elsevier Science Ltd, 2012.

[74] R. Galarini, A. Musco, R. Pontellini, and R. Santi, "Asymmetric synthesis of alkyl 2-arylalkanoates by cross-coupling reactions cabled by pd complexes," *Journal of molecular catalysis*, vol. 72, no. 3, pp. L11–L13, 1992.

[75] M. Ferrer, C. Méndez-García, R. Bargiela, J. Chow, S. Alonso, A. García-Moyano, G. E. Bjerga, I. H. Steen, T. Schwabe, C. Blom *et al.*, "Decoding the ocean's microbiological secrets for marine enzyme biodiscovery," *FEMS microbiology letters*, vol. 366, no. 1, p. fny285, 2019.

[76] V. Brissos, "In vitro evolution of lipolytic enzymes—stabilization of cutinase against surfactants," Ph.D. dissertation, Instituto Superior Técnico, Universidade Técnica de Lisboa, 2006.

[77] M. Shashidhar, J. J. Volwerk, O. H. Griffith, and J. F. Keana, "A chromogenic substrate for phosphatidylinositol-specific phospholipase c: 4-nitrophenyl myo-inositol-1-phosphate," *Chemistry and physics of lipids*, vol. 60, no. 2, pp. 101–110, 1991.

[78] Y. Ni and R. Chen, "Extracellular recombinant protein production from *Escherichia coli*," *Biotechnology letters*, vol. 31, no. 11, p. 1661, 2009.

[79] W. Sattler, H. Esterbauer, O. Glatter, and W. Steiner, "The effect of enzyme concentration on the rate of the hydrolysis of cellulose," *Biotechnology and bioengineering*, vol. 33, no. 10, pp. 1221–1234, 1989.

[80] P. Fernandes, "Unpublished data," 2020.

[81] C. I. Butre, S. Sforza, H. Gruppen, and P. A. Wierenga, "Determination of the influence of substrate concentration on enzyme selectivity using whey protein isolate and *Bacillus licheniformis* protease," *Journal of agricultural and food chemistry*, vol. 62, no. 42, pp. 10 230–10 239, 2014.

[82] M. Z. Kamal, P. Yedavalli, M. V. Deshmukh, and N. M. Rao, "Lipase in aqueous-polar organic solvents: Activity, structure, and stability," *Protein Science*, vol. 22, no. 7, pp. 904–915, 2013.

[83] R. Brancal, "Unpublished work performed in the IBB laboratory," 2020.

[84] F. Carvalho, "Unpublished data," 2019.

[85] K. Kovarova, A. Zehnder, and T. Egli, "Temperature-dependent growth kinetics of escherichia coli ml 30 in glucose-limited continuous culture." *Journal of bacteriology*, vol. 178, no. 15, pp. 4530–4539, 1996.

[86] PreSens Precision Sensing GmbH. (2020) Oxodish® od24. Acessed 16th November 2020. [Online]. Available: https://www.presens.de/products/detail/oxodish-od24

[87] PreSens Precision Sensing GmbH. (2020) Sdr sensordish® reader basic set. Acessed 16th November 2020. [Online]. Available: https://www.presens.de/products/detail/sdr-sensordish-reader-basic-set

[88] M. P. Marques, K. Walshe, S. Doyle, P. Fernandes, and C. C. C. R. Carvalho, "Anchoring high-throughput screening methods to scale-up bioproduction of siderophores," *Process Biochemistry*, vol. 47, no. 3, pp. 416–421, 2012.

[89] C. Coscolín, M. Martínez-Martínez, J. Chow, R. Bargiela, A. García-Moyano, G. E. Bjerga, A. Bollinger, R. Stokke, I. H. Steen, O. V. Golyshina *et al.*, "Relationships between substrate promiscuity and chiral selectivity of esterases from phylogenetically and environmentally diverse microorganisms," *Catalysts*, vol. 8, no. 1, p. 10, 2018.

A

Calibration data

A.1 Biomass

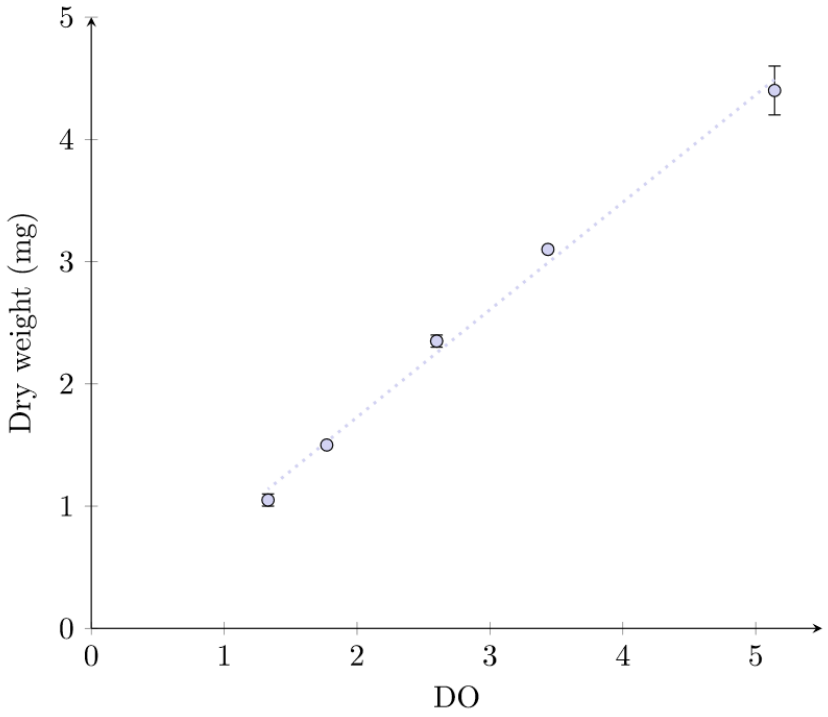


Figure A.1: Calibration curve for dry weight against OD at 580 nm measured using a Thermo Scientific® Multiskan® GO spectrophotometer acquired from Fisher Scientific (Waltham, MA, USA) using a glass cuvette with a 10 mm light path. Dry weight (mg) = 0.8790 $\times$ DO - 0.030. R^2=0.9947.

A.2 MPP

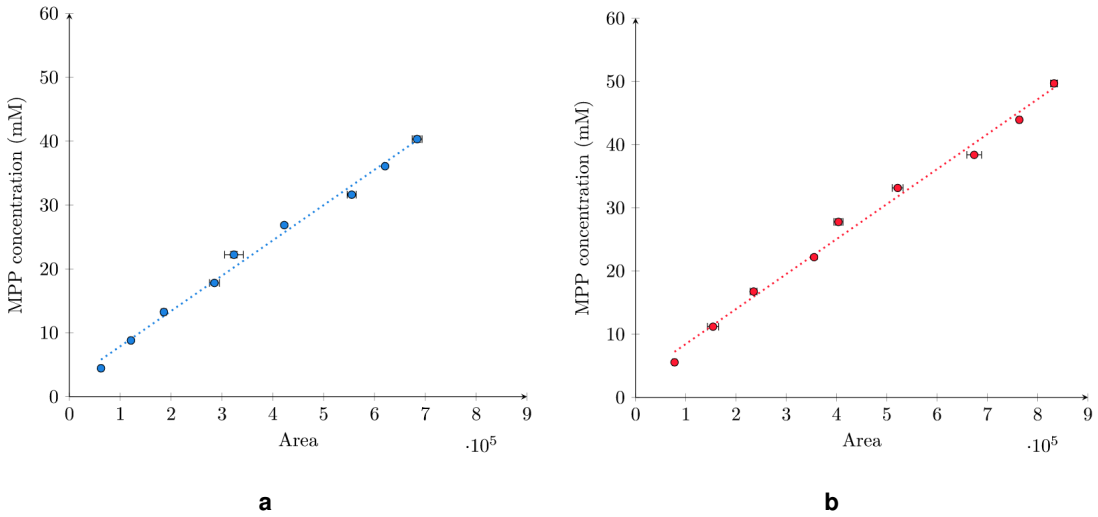


Figure A.2: Calibration curve for the concentration of the *S* and *R* enantiomers of MPP for concentrations between 10 and 90 mM against the peak area measured using a GC-2010 Plus chromatograph acquired from Shimadzu (Kyoto, Japan). This gas chromatograph was equipped with a flame ionization detector (FID-2010 Plus), an AOC-20i auto injector and an AOC-20s auto sampler. The column used was a 25 m CP-Chirasil-Dex CB with an inner diameter of 0.25 mm and a film thickness of 0.25 μm acquired from Agilent (Santa Clara, CA, USA). [S] (mM) = 5.526· 10^{-5} × Area+2.363·. R^2=0.9918 a. [R] (mM) = 5.533· 10^{-5} × Area+2.922·. R^2=0.9905 b.

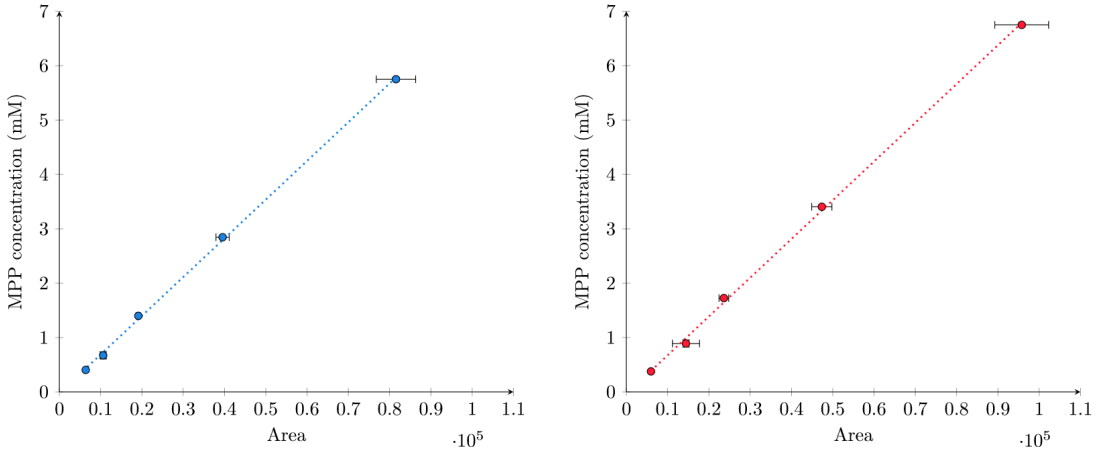


Figure A.3: Calibration curve for the concentration of the *S* and *R* enantiomers of MPP for concentrations between 0.78 and 12.5 mM against the peak area measured using GC-2010 Plus chromatograph acquired from Shimadzu (Kyoto, Japan). This gas chromatograph was equipped with a flame ionization detector (FID-2010 Plus), an AOC-20i auto injector and an AOC-20s auto sampler. The column used was a 25 m CP-Chirasil-Dex CB with an inner diameter of 0.25 mm and a film thickness of 0.25 µm acquired from Agilent (Santa Clara, CA, USA). [S] (mM) = $7.121 \cdot 10^{-5} \times$ Area-$2.523 \cdot 10^{-2}$. R^2=0.9994 a. [R] (mM) = $7.127 \cdot 10^{-5} \times$ Area-$3.843 \cdot 10^{-2}$. R^2=0.9991 b.

B

Growth media

Table B.1: Growth medium for pre-inocula *E. coli* BL21 (DE3) pET22b_Lip_2.

Component	Concentration
LB medium	
Glucose	0.5 %(m/v)
Ampicillin	100 μg/mL

Table B.2: Induction medium for *E. coli* BL21 (DE3) pET22b_Lip_2.

Component	Concentration
Na_2HPO_4	3.1 g/L
KH_2PO_4	1.7 g/L
Tryptone	20 g/L
Yeast extract	5 g/L
NaCl	5 g/L
Ampicillin	100 μg/mL
Glucose	0.05 %(m/v)
Glycerol	0.6 %(v/v)
Lactose	0.2 %(m/v)

C

Oxygen Profiles obtained using the PreSens system

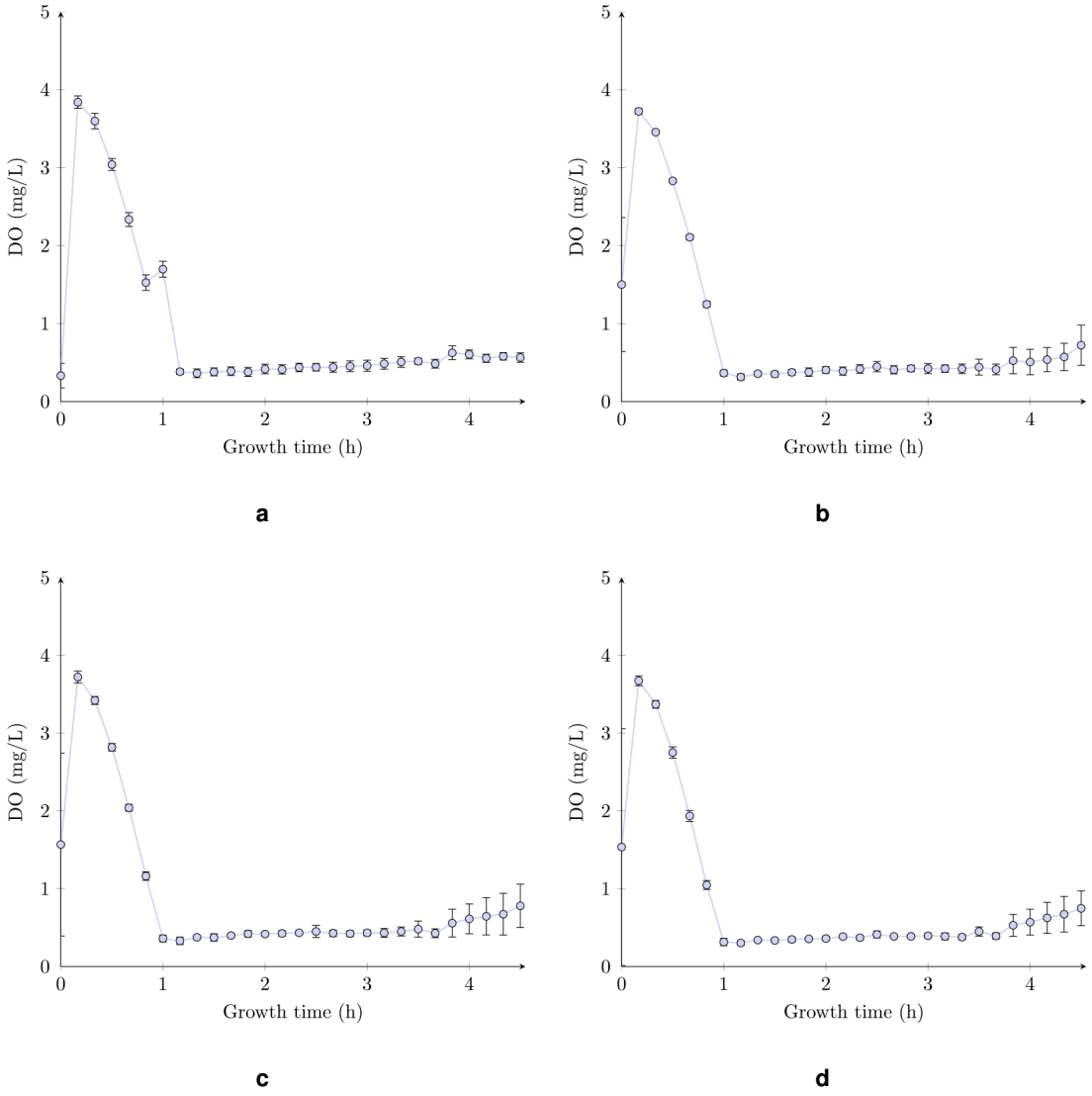


Figure C.1: Oxygen profile measured with the SDR SensorDish® Reader in the well of an a OxoDish® OD24 micro titer plate for *E. coli* BL21 (DE3) pET22b_Lip_2 using the standard medium supplemented with: a 0.05 %(m/v) glucose, 0.6 %(v/v) glycerol and 0.2 %(m/v) lactose; b 0.5 %(m/v) glucose, 0.6 %(v/v) glycerol and 0.2 %(m/v) lactose; c 0.75 %(m/v) glucose, 0.6 %(v/v) glycerol and 0.2 %(m/v) lactose; and d 1 %(m/v) glucose, 0.6 %(v/v) glycerol and 0.2 %(m/v) lactose at 30 °C and 160 rpm.

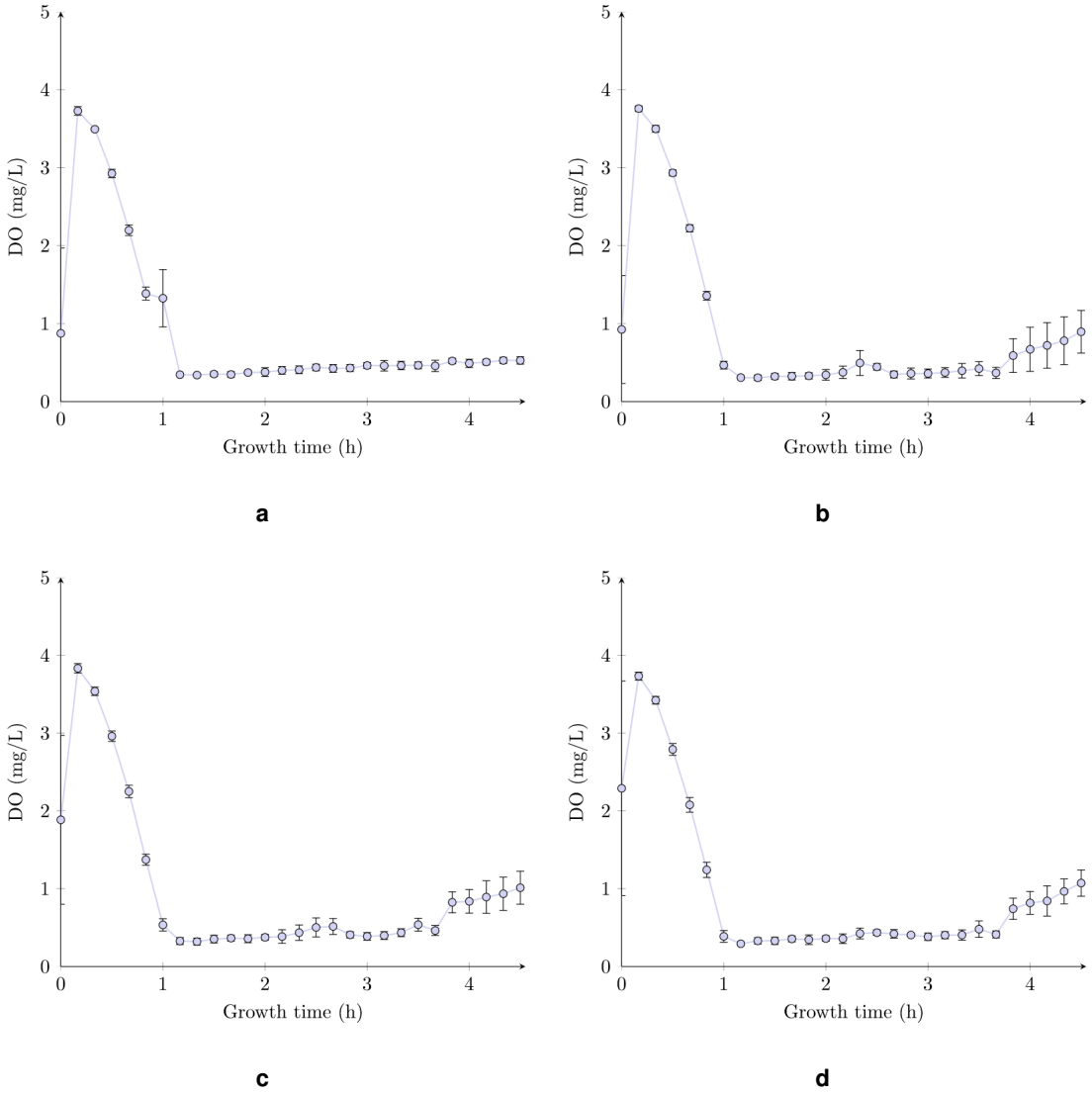


Figure C.2: Oxygen profile measured with the SDR SensorDish® Reader in the well of an a OxoDish® OD24 micro titer plate for *E. coli* BL21 (DE3) pET22b_Lip_2 using the standard medium supplemented with: a 0.05 %(m/v) glucose, 0.6 %(v/v) glycerol and 0.5 %(m/v) lactose; b 0.5 %(m/v) glucose, 0.6 %(v/v) glycerol and 0.5 %(m/v) lactose; c 0.75 %(m/v) glucose, 0.6 %(v/v) glycerol and 0.5 %(m/v) lactose; and d 1 %(m/v) glucose, 0.6 %(v/v) glycerol and 0.5 %(m/v) lactose at 30 °C and 160 rpm.

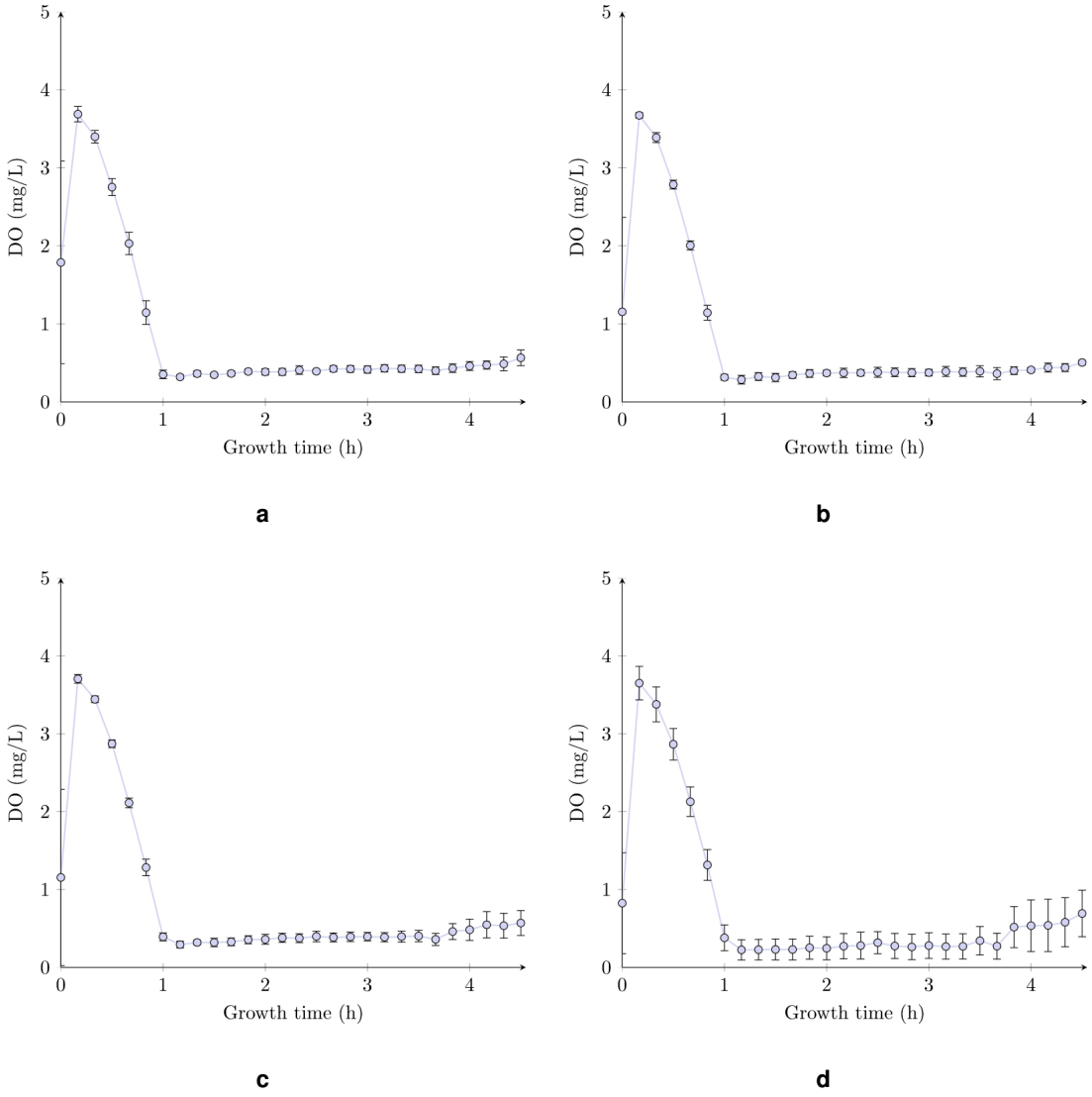


Figure C.3: Oxygen profile measured with the SDR SensorDish® Reader in the well of an a OxoDish® OD24 micro titer plate for *E. coli* BL21 (DE3) pET22b_Lip_2 using the standard medium supplemented with: a 0.5 %(m/v) glucose, 0.05 %(v/v) glycerol and 0.2 %(m/v) lactose; b 0.5 %(m/v) glucose, 0.1 %(v/v) glycerol and 0.2 %(m/v) lactose; c 0.5 %(m/v) glucose, 0.25 %(v/v) glycerol and 0.2 %(m/v) lactose; and d 0.5 %(m/v) glucose, 0.8 %(v/v) glycerol and 0.2 %(m/v) lactose at 30 °C and 160 rpm.

D

Equipment

Table D.1: Stirring speed (rpm) on each spot of the Variomag Poly 15 Multi-Point Inductive-Drive Stirrer (Thermo Scientific, Waltham, MA, USA) used in this work when set to 600 rpm. Spots are indentified in superscript.

^{1}599.7	^{2}600.0	^{3}599.8
^{4}600.4	^{5}598.0	^{6}599.1
^{7}600.8	^{8}598.0	^{9}599.1
^{10}600.6	^{11}600.0	^{12}600.8
^{13}600.2	^{14}599.6	^{15}600.0