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

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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 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 an 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 *(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

1. Introduction

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.

The thalidomide disaster in the late 1950s, was responsible for limb malformations across several countries in tens of thousands of victims. In thalidomide the *R*-enantiomer is therapeutic while the *S*-enantiomer is teratogenic [1, 2]. 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 EU) and the Kefauver-Harris drug amendments in 1962 in the United States of America [3, 4]. 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. Similar guidelines were put into place by the Food and Drug Administration in the United States of America in 1992 [5, 6].

Ideally the best ways to procure an enantiopure substance are to isolate it directly from a natural source or to synthesize it directly. However not everything is available from natural sources and asymmetrical synthesis can be out of reach due to the high cost or outright unavailability of the necessary chiral catalysts. Therefore the resolution of a mixture of enantiomers can be necessary.

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. Enzyme catalysts attract a high amount of attention because of their high specificity and the possibility of mild reaction conditions [7]. When using enzyme catalysts, ideally only one of the enantiomers reacts, and the reaction can reach total conversion. Among the vast amount of enzymes, lipases have been the most widely reported in kinetic resolution due to their availability and wide substrate range [8].

Lipases (tryacylglycerol lipases - EC 3.1.1.3) catalyse the cleavage of a C-O ester bond (Figure 1) and have a very high substrate variability [9]. Due to the high substrate variability ester

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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 [10, 11]; in the synthesis of fine chemicals [12]; as detergents [13]; in bioremediation of waste [14] and in the production of biodiesel [15].

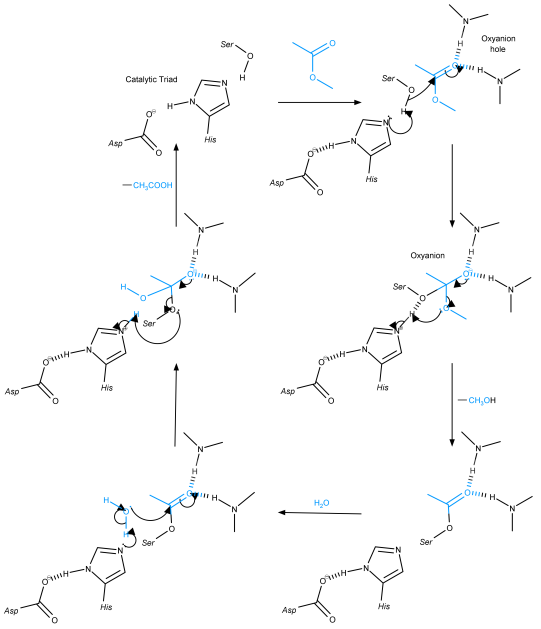


Figure 1: Cannonical enzyme catalysed ester hydrolysis mechanism. Reaction scheme for the hydrolysis of methyl ethanoate.

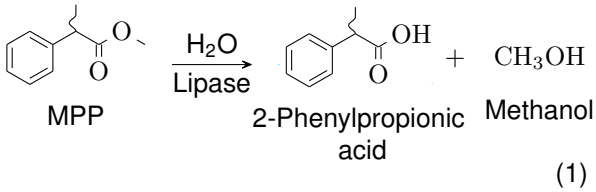
The search for cleaner industrial processes led to the creation of the Industrial Applications for Marine Enzymes (INMARE) (grant agreement number 634486) project which brought together industry and academia partners to facilitate the process of developing new commercially significant enzymes (`http://www.inmare-h2020.eu/`). This project succeeded in decreasing the average time in developing useful 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 [16].

Due to regulatory mandated necessities pharmaceutical companies must assess the effect of each enantiomer in chiral drugs, so for kinetic resolution assays INMARE partners provided methyl 2-phenylpropanoate (MPP) as a model substrate 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 non-steroidal anti-inflammatory drugs.

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).

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 (Equation 1) 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. The enzyme Lip_1 was found to selectively hydrolyse the *S*-enantiomer of MPP, while the lipase Lip_2 was found to selectively hydrolyse the *R*-enantiomer of MPP.



(1)

2. Materials & Methods

2.1. Reagents

MPP was provided by a partner of the INMARE project, as a mixture of the *S*- and *R*- enantiomers at 45.1 % and 54.9 % (molar), respectively (ee of 9.8 %). D-(+)-Glucose (≥99.5 %) and 4-Nitrophenyl butyrate (p-NPB) (≥98 %) 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), DMSO (≥99.7 %), and glycerol (99.96 %) were purchased from Fisher Scientific (Waltham, MA, USA), sodium chloride (≥95 %) and hydrochloric acid (≥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), 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 and bacterial strains

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

2.3. Bacterial growth

The cellular growths were performed in Erlenmeyer flasks. The composition of the growth media is available at the Appendix - Tables 3 and 4. 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 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. Enzymatic assays

2.4.1 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 [17]). One unit of enzymatic activity - U (p-NPB) - was defined as one micromole of p-NPB hydrolysed per minute.

2.4.2 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(tetrafluoroethylene) 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). 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 tubes and on a 5810 R centrifuge (from Eppendorf) for the 5 mL and 15 mL tubes.

2.5. Analytical methods

2.5.1 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.

2.5.2 Methyl 2-phenylpropanoate analysis

MPP concentration in hexane was measured by gas chromatography on a GC-2010 Plus GC from Shimadzu (Kyoto, Japan). This 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 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.

2.6. 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.

$$ee\ (\%) = \frac{[e_1] - [e_2]}{[e_1] + [e_2]} \times 100 \quad (2)$$

In this work e_1 represents the *R*-enantiomer, while e_2 represents the *S*-enantiomer of MPP.

2.7. Enzyme quantification

The total amount of enzyme added in each of the MPP hydrolysis assays was quantified through enzymatic activity on p-NPB hydrolysis. 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.

The activity for p-NPB hydrolysis was determined using the Lambert-Beer law (Equation 3), 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 1).

$$\frac{dA}{dt} = \varepsilon l \frac{dC}{dt} \quad (3)$$

Table 1: Values for the constants ε and l used in the Lambert-Beer law for the system in use.

Molar absorptivity (ε)	15.4×10^3 $M^{-1}cm^{-1}$ [18]
Light path length (l)	1 cm

3. Results & discussion

3.1. 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, Lip_2 is an intracellular enzyme (Table 2) [19].

Table 2: 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†

3.2. Lip_1 characterisation

3.2.1 Effect of enzyme and substrate concentration

Increasing Lip_1 from the concentration corresponding to 3.25 U to 19 U (p-NPB) led to a 4.92-fold increase in Lip_1 hydrolytic activity in MPP, however when increasing Lip_1 to the concentration corresponding to 26 U (p-NPB) a 7.85 % decrease in Lip_1 hydrolytic activity was observed (data not shown). Lip_1 activity was expected to plateau for high enzyme concentrations [20], and no support for this behaviour was found in the literature, however similar anecdotes were found [21]. For the following assays a Lip_1 concentration corresponding to 13 U was chosen due to Lip_1 activity still increasing with the enzyme concentration.

An increase in substrate concentration from 20 to 100 mM naturally led to an increase in Lip_1 selectivity due to increased availability of the preferred enantiomer (data not shown). A substrate concentration of 40 mM was chosen for the following assays.

3.2.2 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 [22]. In this work the effect of methanol and DMSO on the activity of the enzymes was assessed.

Methanol at 10 % (v/v) had 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 2b). 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 2b).

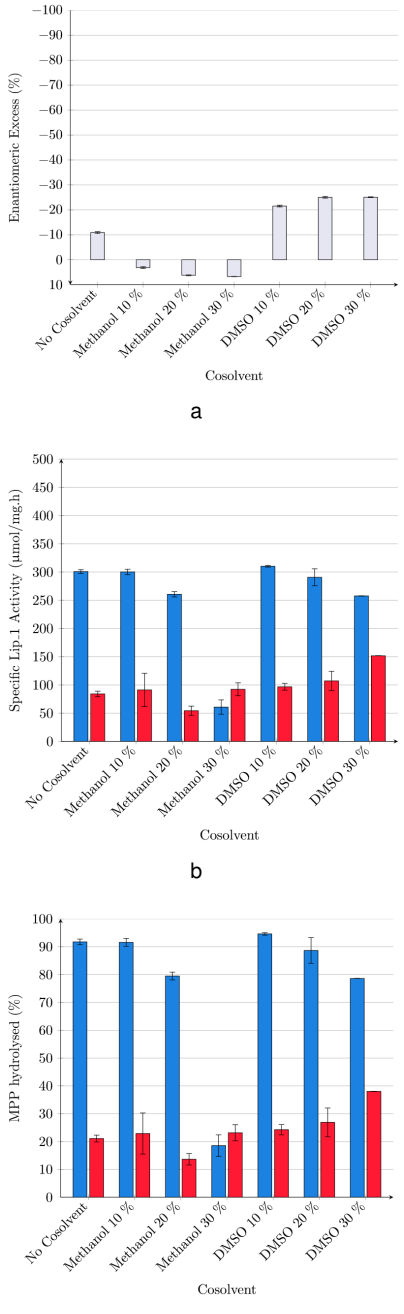


Figure 2: Effect of the presence of methanol and DMSO in the reaction media at 10, 20 and 30 %(v/v) on: a the enantiomeric excess of MPP; b specific activity of Lip_1 on the *S* (blue) and *R* (red) enantiomers of MPP; and c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed during 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.

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 2c 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.

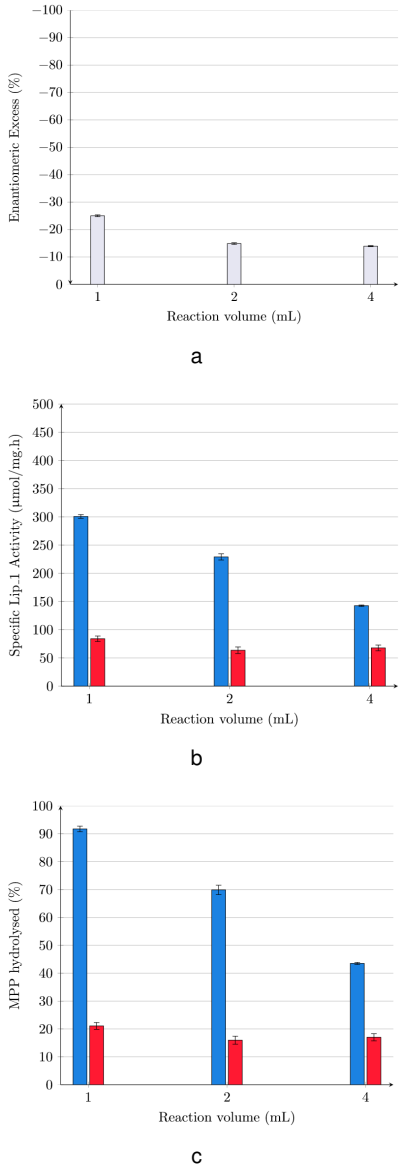


Figure 3: Effect of doubling and quadrupling the reaction volume on: a the enantiomeric excess of MPP; b specific activity of Lip_1 on the *S* (blue) and *R* (red) enantiomers of MPP; and c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed during the reaction. The enzyme concentration 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.

3.3. Bioreaction run

Lip_1 selectivity decreased as expected through the reaction due to the relative abundance of enantiomers shifting toward the non-preferred *R*-enantiomer. After 1 h enantiomerically pure *(R)*-MPP was achieved with a specific Lip_1 activity of 272.7 µmol MPP/mg.h and a yield of 45.4 % (data not shown).

3.3.1 Scale-up

When increasing the reaction volume from 1 mL to 4 mL a clear reduction of the selectivity was ob-

served (Figure 3). 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 b), which caused a reduction on the conversion of (S)-MPP (Figure 3c) and on the enantiomeric excess (Figure 3a). 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 [23].

3.4. Lip_2 characterisation

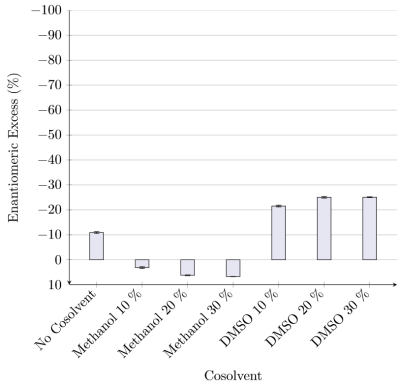
3.4.1 Effect of enzyme and substrate concentration

Increasing Lip_2 from the concentration corresponding to 0.61 U to 4.88 U (p-NPB) led to a decrease of the ee of MPP from -7.51 % to -95.3 %, the increase to -86.0 % and -76.5 % when using Lip_2 concentrations corresponding to 9.76 and 14.64 U (p-NPB), respectively is due to a decrease in Lip_2 activity on the *R*-enantiomer of MPP (data not shown). This is also likely due to mass transfer limitations. Like previously mentioned the expected behaviour is a plateau [20]. For the following assays a Lip_2 concentration corresponding to 1.22 U was chosen due to Lip_2 activity still increasing with the enzyme concentration.

An increase in substrate concentration from 20 to 100 mM led to an increase in Lip_2 activity. From 60 mM to 80 mM MPP concentration there was a drastic 2.7-fold increase in Lip_2 activity (data not shown). Since this assay was performed using whole cells this can be explained by increased MPP diffusion into the cells. A substrate concentration of 60 mM was chosen for the following assays.

3.4.2 Co-solvent effect on Lip_2 activity

Methanol had a negative effect on Lip_2 enantioselectivity, reducing 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 4b).



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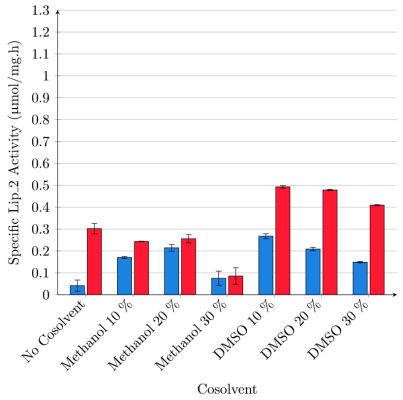

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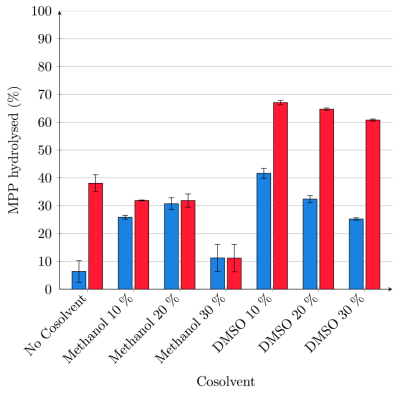

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Figure 4: Effect of the presence of methanol and DMSO in the reaction media at 10, 20 and 30 %(v/v) on: a the enantiomeric excess of MPP; b specific activity of Lip_2 on the *S* (blue) and *R* (red) enantiomers of MPP; c and percentage of the *S* (blue) 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.

The following assays were done using a 20% DMSO concentration because of the increased Lip_2 activity and balance between conversion and ee (Figures 4a and c).

3.4.3 Bioreaction run

Lip_2 activity greatly decreased through the reaction, being only 6.71 % of the initial activity left after 24 h. After 24 h Lip_2 had already lost its selectivity (data not shown). Not even after 24 h of reaction it was possible to obtain enantiopure *S*-MPP.

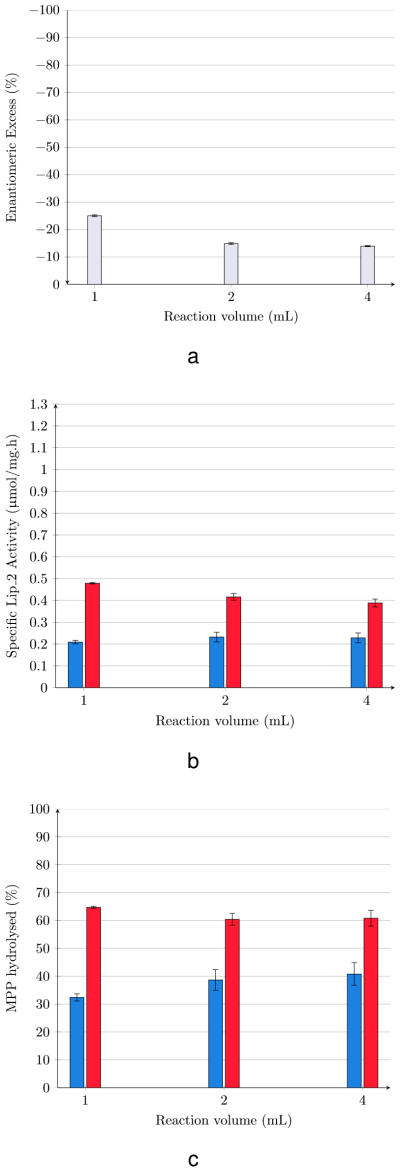

Figure 5: Effect of doubling and quadrupling the reaction volume on: a the enantiomeric excess of MPP; b specific activity of Lip_2 on the *S* (blue) and *R* (red) enantiomers of MPP; and c percentage of the *S* (blue) and *R* (red) enantiomers of MPP hydrolysed during the reaction. The enzyme concentration 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.

3.4.4 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 5b) which led to reduced ee and *(R)*-MPP conversion (Figures 5a 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 [23].

4. Conclusions & future work

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 low enantioselectivity [24]. 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.

Due to time constraints caused by the COVID-19 pandemic, the proposed reaction systems were not completely studied. In order to alleviate the mass transfer limitation problems found during the work cell different cell disruption methods were supposed to have been tried, the ideal candidates being high pressure homogenization and chemical disruption through the addition of an easily separable organic solvent due to their possibility of large scale application. Enzyme immobilization, a request of the INMARE industry partners, was also not performed. Enzyme immobilization has been shown to greatly influence enzymatic activity and stability, while also making the immobilized

enzyme easier to separate from the reaction mixture than free enzymes.

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.

5. Acknowledgements

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Appendix

Table 3: 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 4: 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)