Stability and enantioselectivity of selected marine enzymes

Ricardo Damman Rosa Duarte Brancal

IBB-Institute for Bioengineering and Biosciences

Master's degree in Biological Engineering, Instituto Superior Técnico, Lisbon, Portugal

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Abstract

Biocatalysis allows the synthesis of compounds in a sustainable way and can replace chemical catalysis in several processes. Biocatalysis, or catalysis mediated by enzymes, has many applications in many types of industries, such as the pharmaceutical, leather, and starch industry. The present work aimed at studying the effect of the storage temperature on the activity of the selected enzymes and understanding the factors influencing both activity and enantioselectivity of the enzymes in bioreactors with different volumes. The biocatalytic performance of two esterases from marine microorganisms origin with potential industrial application as biocatalysts, was assessed in bioreactors with 3 volumes to assess which were the most important parameters when designing a successful scale-up strategy up to industrial scale. The enantioselective resolution of the racemic substrate methyl-2-phenylpropanoate was performed with the enzyme esterase EH in an organic: aqueous system in the 3 volumes, 10, 20 and 40 mL. Variations in the mixing time and interfacial area when the reactor volume was increased, were responsible for the results of the EH enantioselectivity differences towards the substrate methyl-2-phenylpropanoate. Both activity and stability of the EH and Abo enzymes towards the substrate *p*-nitrophenyl butyrate were assessed at different storage temperatures, -16ºC and +4ºC. Several experiments initially planned for this work, could not be conducted due to COVID-19. This work was carried out with two esterases provided by the partners of the "INMARE" project

Keywords: Enzyme, biocatalysis, enantioselectivity, bioreactor

1. Introduction

The study of marine biocatalysts on a global scale is still on its early phase, since the marine environment possesses an untapped huge potential for the development of new industrial applications with several benefits due to marine biological diversity and to the specificity of marine metabolisms. The pool of marine biodiversity is an excellent reservoir for acquiring an inventory of enzymes that is of the focal points of the potential of blue technology, some important examples of enzymes are among oxireductases and carbohydrate-active enzymes[9].

Marine enzymes have unique properties that can provide several benefits to many industrial sectors such as the pharma sector. Nonetheless most of the marine microbiome cannot be cultured, which leads to under exploration of the vast enzymes pool potential[10]. Marine environment possesses a vast resource for the pharmaceutical industry, and marine microorganisms such as bacteria and fungi, that adapt to the most extreme environmental conditions, such as low temperature, high salinity, and extreme pressure, have developed particular metabolites that allow them to survive, proliferate and turnover of key biological elements. For example, the survival of some bacteria in the harsh marine environment is dependent on the production of biologically active compounds such as biosurfactants, siderophores, specialized fatty acids and terpenoids[11]. These microorganisms can be used for a variety of biotechnological applications. For example, organisms that thrive in permanently cold habitats, like Polar zones, are full of cold-adapted enzymes that can have a variety of industrial applications, such as ingredient in cleaning agents, in the biodegradation of xenobiotic compounds in cold areas, in food processes like the cheese manufacturing and meat tenderization[12][13].

Lipases and esterases in particular, can be suited for a range of industrial applications, due to their stability, enantio-/stereoselectivity and their stability and high levels of activity in organic solvents[14]. Regarding wastewater management, catalysts can facilitate easy and rapid reduction of complex substances such as fats and oil. Lipases from microbial origin, could in fact reduce the fats and oil contents in wastewater effluents. Also esterases can have an important role in the biodegradation of natural substances in wastewater including lipids, and also in a range of synthetic chemicals by hydrolysing short chain esters, whereas lipases can hydrolyze longer chain esters[15]. The screening of environmental sources like the ocean, is a very promising tool for the identification of new biocatalysts with synthetically useful properties[16].

2. Materials and methods

2.1. Enzymes and Reagents

The esterase EH was gently provided by the group of Dr. Manuel Ferrer of CSIC, Spain, in lyophilized cells and the esterase Abo was gently provided in wet cell extracts by the group of Prof. Karl-Erich Jaeger of the Heinrich Heine University Düsseldorf, Germany. Both were provided in the context of project INMARE, funded by the EU Horizon 2020 programme. The substrate methyl-2-phenylpropanoate (MPP) was gently provided by Dr. Rainhard Koch. 4-Nitrophenyl butyrate (p-NPB), ethanol, acetone, Ethanol, and crystal violet were obtained from Sigma Aldrich. NaOH, HCl, and hexane were obtained from Fischer Chemicals.

2.2. Enzymatic resolution

The activity of the enzymes provided by the partners of the "INMARE" project, were tested with both substrates, *p*-NPB, and MPP in 3 reactor volumes with 10, 20, 40mL under different reaction conditions. Enantioselectivity of EH was evaluated towards a racemic mixture of MPP, also on the 3 reactors.

The first step was to characterize the reaction velocity across a range of substrate concentrations, using a colorimetric spectrophotometric assay. The cuvette used had a 1,5 mL of working volume composed by Tris-HCl pH 8 buffer, *p*-NPB, and the biocatalyst (EH or Abo). The results of this assay were plotted, where the velocity was represented by (umol/min*g cell) and the substrate concentration in (mM/ml), to then obtain the reaction kinetic parameters, Km and Vmax.

The following step was to evaluate the reactor volume increase effect on the hydrolysis of the substrate *p*-NPB. For that, each reactor was filled with 25% of its total volume, meaning for example that a reactor with a total volume of 10mL, would have a reaction volume of 2.5mL. The substrate and enzyme concentration remained constant at all scales (Table 1).

Table 1 – Description of the enzyme, substrate and buffer volumes and concentration for the hydrolysis assay of the substrate, *p*-NPB

Scale	**10 mL**	**20 mL**	**40 mL**
Working volume	2.5 mL	5 mL	10 mL
Abo mass of cells	$4.2x10^{-6}$ g cells	$8.4x10^{-6}$ g cells	$16.8x10^{-6}$ g cells
EH mass of cells	$5.0x10^{-7}$ g cells	$10.0x10^{-7}$ g cells	$20.0x10^{-7}$ g cells
***p*-NPB volume**	0.035 mL	0.070 mL	0.140 mL
***p*-NPB concentration**	79.64mM	79.64mM	79.64mM
Tris HCl 100 mM pH 8 volume	2.44 mL	4,88 mL	9,76 mL

Reaction time was 30 minutes for all reactor volumes, with samples of 200 µL taken directly from the reactor to the cuvette, every 3 minutes. The cuvette had a total volume of 1 mL, where 800 µL were water milli Q and 200 µL were samples from the reactor.

The experiment was tested under light or dark conditions and with or without heating. This means that the reactors would be covered or not by aluminium foil, and then, for the temperature control they would be involved in an immersion thermostat (LAUDA E100) programmed for 30ºC, otherwise, they would be at 22ºC, approximately 22ºC.

2.2.1 Biphasic system

The biphasic system was composed of an aqueous and an organic phase. A 200 µl solution in a GC vial was prepared with the following components: the amount of mehyl-2-phenylpropanoate to reach 40, 60, 80, 100, and 160 mM methyl-2-phenylpropanoate; 21, 42, and 84 µL of EH17 for the 10, 20, and 40mL scale respectively in Tris-HCl 100mM pH 8 buffer. These solutions were incubated at 30ºC and 800 rpm in an Agitorb 200 incubator for 5 hours for an MPP concentration of 40mM, and 6 hours for the remaining concentrations (60,80,100 and 160 mM). During the reaction course, samples were periodically taken as suicide assays and analyzed by chiral-GC.

2.2.2 Freezing and thawing cycles influence on the stability of the studied enzymes

Both biocatalysts activity was assessed when submitted to different storage temperatures for approximately 90 days. EH and Abo were stored in the freezer between at -18ºC, and in the refrigerator between at +4ºC. The enzymes suspensions used during the experiments were always the same, which means that the same enzymatic suspension was used again after each freezing/thawing cycle. To test this, 1mL of a mixture enzyme, buffer, and substrate were prepared on a 2mL size cuvette to then measure the biocatalyst activity on a spectrophotometer Hitachi U2000 with agitation of 600rpm. For EH activity assay, the mixture was composed of: 1470µL of Tris-HCL 20mM buffer + 15µL of an EH solution diluted in the ratio of 1/20 in Tris-HCl 100mM buffer (initial EH suspension was composed by 10mg dry cells for 1mL of Tris-HCL 100mM buffer) + 15µL of *p*-NPB . For Abo activity assay, the mixture was composed of: 1470µL of Tris-HCL 20mM buffer + 15µL of an Abo solution diluted in the ratio of 1/5 in Tris-HCl 100mM buffer (initial Abo suspension was composed by 1mg dry cells for 10mL of Tris-HCL 100mM buffer). It should be noted that before the enzyme assay was performed, all reagents, enzymes, and buffer where thermostabilized at 24ºC

2.2.3 Reactors mixing efficiency

The mixing efficiency at the 3 reactor volumes was evaluated by applying a tracer to the reaction solution that was the same used for the analysis of the enantioselectivity assay. The experiment consists of recording a video of the mixing process, to extract the color changes for 360 seconds. The video was captured by a digital camera (avi format, 60 frames per second) The tracer used was Crystal Violet from Fisher Chemical at a concentration of about $1x10^{-5}M$[7].

The time that the solution took to became transparent was measured and then the data was evaluated by an image analysis software (ImageJ). The aim was to evaluate the time that was needed for the reaction solution to become transparent by detecting the inflection point of the graph where the intensity of the channels was plotted against time. For that reason, the color change is quantified employing individual thresholds on the RGB (Red, Green, and Blue) color model and provides a direct measurement of the mixing efficiency.

2.2.4 Interfacial area

The interfacial area was also evaluated for all scales. For that experiment, 3 reactions were prepared for all 3 scales in duplicate, and the samples (50µL) were taken at approximately 25 % and 50% substrate conversion (Table 2).

Table 2 – Sampling times for 25% and 50% of substrate conversion across reactor volumes

Scale (mL)	Reaction Time at 25% substrate conversion (h)	Reaction Time at 50% substrate conversion (h)
10	0.55	1.77
20	0.77	2.15
40	0.93	2.37

The chosen criteria to measure the residence time was the substrate bubble diameter size distribution across the bioreactor scales. The images collected with a 1500x magnification (10x ocular and 150x objective) were processed in "Fiji", with a total image area of $4.8x10^5 \mu m^2$. To confirm the software validity, each image was analyzed manually to confirm bubble sizes and their frequency.

2.3. Bioreactors volumes and geometries

Reactors used for the experiments were acquired from Phenomenex. Information of the reactors models and screw caps is provided in tables 3 and 4. The magnetic stirrer bar used for all the reactors was acquired from Kartell, with a length of 12 mm and a diameter of 3mm.

Table 3 - Verex vials dimensions and characteristics provided by the manufacturer -Phenomenex

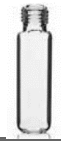
VEREX vials			
Total Volume	10 mL	20 mL	40 mL
Dimension	23 x 46 mm	23 x 75 mm	28 x 95 mm
Neck	18 mm	18 mm	24 mm

Table 4 - Verex screw caps dimensions and characteristics provided by the manufacturer – Phenomenex

Screw Caps		
Designation (*certified caps)	18 mm (Magnetic Caps for Headspace)	24 mm (24-414)
Vials compatibility	10, 20 mL	40 mL

2.4. Enzymatic activity

2.4.1 Enzymatic activity towards *p*-nitrophenyl butyrate

The volume of the sample containing the enzyme, buffer, and substrate under study were changed to have more data for a better enzymatic activity assay evaluation. Also, if there was a sample with a very high activity whose reading could not be done on the spectrophotometer, the prioritized criteria would be to decrease the enzyme concentration on the solution either by previously diluting the enzyme or by decreasing the enzyme volume in the sample. The absorbance was recorded at a wavelength of 410 nm (ε_{410}=15.4×103 $M^{-1}cm^{-1}$ in water), while the reaction system was kept at 30°C and mixing at approximately 600rpm. One unit of enzymatic activity is equivalent to the conversion of 1 µmol of p-NPB per minute.

The enzymatic activity was determined from the happening of a hydrolysis reaction of the ester group on p-NPB, as shown in figure 1. The reactions resulting solution showed a yellow colour [8].

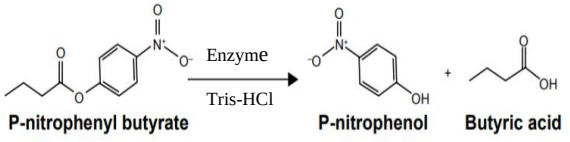


Figure 1 – Hydrolysis of *p*-nitrophenyl butyrate (adapted from [17])

For the determination of the enzymatic activity, the Lambert-Beer law was applied, which is represented in equation 6, where the absorbance is relative to the concentration of the product obtained, in this case, p-nitrophenol (*p*-NP).

$$\frac{dAbs}{dt} = \frac{\varepsilon \times 1 \times d[product]}{dt} \quad (1)$$

2.3.2 Enzymatic hydrolysis of methyl-2-phenylpropanoate

The enantioselectivity of the biocatalyst EH17 towards a racemic mixture of methyl-2-phenylpropanoate was assessed by recurring to chiral gas chromatography, where the variation of each enantiomer of the substrate was monitored. This study aimed to evaluate the effects that the scale-up had on the enantioselectivity of the enzyme where the desired product was the enantiopure substrate that would not be transformed by the enzyme and not the product of the enzymatic reaction, i.e, the acid (Figure 2).

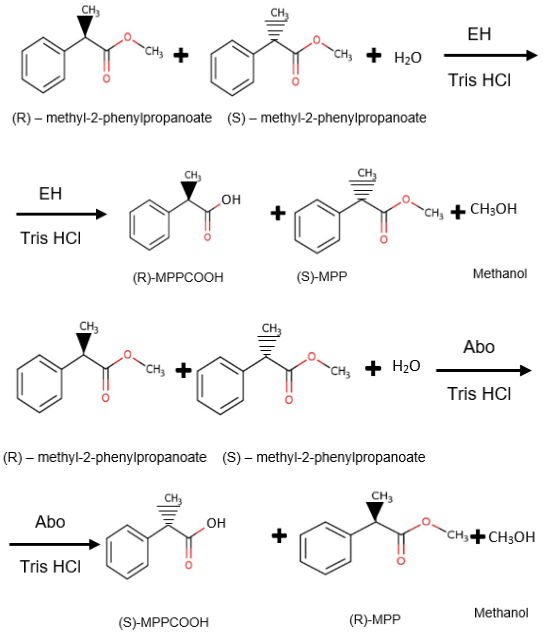


Figure 2 – Hydrolysis of methyl-2-phenylpropanoate to methyl 2-phenylpropanoic acid

2.4. Analytical methods

2.4.1 Samples preparation for the gas chromatography analysis

To perform the enantioselectivity analysis of the EH enzyme toward the racemic mixture of methyl-2-phelylpropanoate, a chiral GC was required. The equipment used was the GC-2010 from Shimadzu, which included a AOC-20s autosampler. The GC was equipped with a 25m long variant capillary column CP-chirasil-DexCB, 0.25mm, and 0.25 µm. The software used to control and program the equipment was the GC solution analysis version 2.30.00 from Shimadzu. In the enzymatic reactions of the biphasic organic system, the reaction volume was 1, 2, and 4mL for the respective 10, 20, and 40mL reactors. When the substrate concentration was 40mM the reaction time was 5h. For the remaining substrate concentrations, 80mM and 160mM, the reaction time was increased to 6h. The volume of the organic solvent added (hexane) corresponded to the reaction volume. So, if the reaction volume was 1mL, it was also added 1mL of the organic solvent.

3. Results and Discussion

3.1. Storage of lyophilized and wet paste cells, how may cycles of freezing and thawing can they hold?

This study aimed to evaluate how many freezing and thawing cycles could the lyophilized EH cells and the Abo in wet paste cell extract hande . This experiment took 84 days for EH enzyme and 91 days for Abo enzyme (Figures 3 and 4 respectively). The enzyme activity is expressed as µmol/min.mg of dry cells.

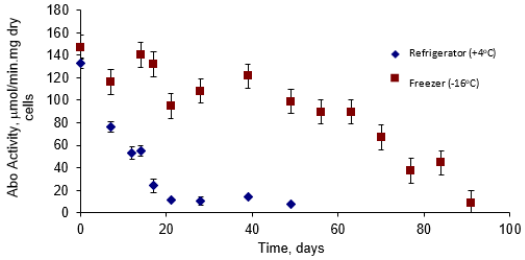


Figure 3 – Abo activity after freezing and thawing cycles

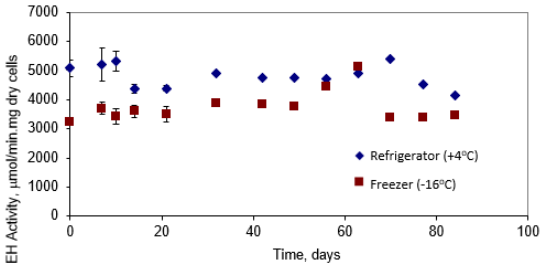


Figure 4 - EH activity after freezing and thawing cycles

According to Figure 3, Abo enzymes decreased their activity after the first freezing/thawing cycle, losing 40% of the initial activity up to the 9th cycle at a storage temperature of -16ºC. For the storage temperature of +4ºC, after 55 days, almost no activity of Abo could be detected, whereas when stored at -16ºC, it took 14 cycles (91 days), for the enzymes to lose their activity.

Figure 4 shows that EH cells maintained their activity even after 13 cycles, at both storage temperatures. For 84 days, EH maintained higher activity at +4ºC when compared to Abo. EH cells, when stored at +4ºC had 17% more activity than when stored at -16ºC. Another result to take into consideration is that EH, when stored in the freezer (-16ºC) despite some slight fluctuations, finishing 84 days with practically the same initial activity.

Ultimately, this study suggests that lyophilized cells conserve better their catalytic activity in comparison to

wet cell paste, possibly to the fact that wet cell paste are most prone to bacterial contaminations than lyophilized cells, even considering that eventually after some hours at a storage temperature of +4ºC, both enzyme samples would be contaminated for example by proteases.

3.2. Biocatalysts activity for the enzymatic hydrolysis of *p*-NPB for 3 reactor volumes

Abo and EH were subjected to dark or light conditions at different temperatures, either 22ºC or 30ºC. The enzymes provided better biocatalytic activities at 30ºC and dark conditions, comparing to 22ºC and light conditions, meaning that hydrolysis of *p*-NPB is better at 30ºC at dark conditions since *p*-NPB is a photosensitive substrate.

Both enzymes lost activity when the reactor volume was increased at 30ºC under dark conditions (Table 5 and Figure 5). Abo lost 21.06 % of its activity between 10 and 20 mL, which was the biggest loss in activity at 30ºC under dark conditions. As it was already mentioned, at 30ºC under dark conditions, the increase in the reactors volume did not caused the same reduction in activity for EH as it did for Abo.

Table 5 – Abo and EH activity at 30ºC under dark conditions

Reactor Volume (mL)	Activity – 30ºC / Dark (EH)	Activity - 30ºC / Dark (Abo)
10	12896.01 U	272.34 U
20	12682.01 U	215.41 U
40	12362.26 U	206.99 U

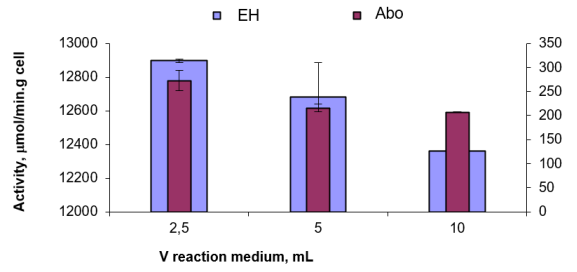


Figure 5 – Activity comparison between both esterases during the enzymatic hydrolysis of *p*-NPB at 30ºC under dark conditions. The activity of the esterases is expressed as mmol/min*g cells

Volume increase at 22ºC under light conditions had opposite effects on the activity of the biocatalysts. EH activity was higher when the reaction volume increased, being the biggest activity difference found between 10 and 20 mL reactors (13.34%), while Abo, lost most of its activity in between the 20 and 40 mL reactors (12.82%) (Table 6 and Figure 6).

Table 6 – Abo and EH activity at 22ºC and dark conditions

Scale (mL)	Activity – 22ºC and dark conditions (EH)	Activity – 22ºC and dark conditions (Abo)
10	7629.87 U	203.32 U
20	8648.17 U	200.29 U
40	9356.55 U	174.61 U

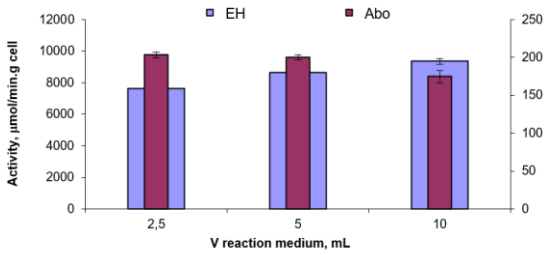


Figure 6 – Activity comparison between both esterases during the enzymatic hydrolysis of *p*-NPB at 22ºC under light conditions. The activity of the esterases is expressed as mmol/min*g cells

This study suggests that both the reaction temperature and light influenced the behavior of the enzymes, regarding their catalytic capacity. It was expected that the implemented heating bath system that standardizes the reaction temperature across the rector volumes at 30ºC, as well as the light barrier provided by the aluminum foil, could mitigate the differences found across the reactors. The results show that the most stable enzyme was EH when at 30ºC under dark conditions, but when the reaction system was at 22ºC under light conditions, the behavior of the enzyme went against expected, presenting higher activity at larger reactor volumes. Regarding Abo activity during reactor volume increase, the difference in activity was bigger at 30ºC under dark conditions. These differences in the biocatalyst's activity, even at controlled reaction temperatures under dark conditions was not expected. That was the motivation to understand the reaction conditions influencing the difference in activity between the reactor volumes, and the motivation for following studies regarding reactors mixing efficiency and interfacial area.

3.2. Reactors mixing efficiency

In the context of this work, this experiment aimed to find if the previous differences in activities across the reactor's volumes could be explained by differences in the reactors mixing efficiency. The mixing efficiency was characterized by the time that the mixture inside each reactor, needed to become completely transparent. The faster the reaction mixture became transparent, the faster crystal violet reacted with NaOH, which reflects on the reactor's mixing efficiency. The reaction volumes were 1, 2 and 4mL for the 10, 20 and 40 mL reactors respectively, with a magnetic agitation at 900 rpm, for 360 seconds. The data for the RGB channels

were plotted into a linear regression by fitting a linear equation to the observed data. Figure 7 represents the Stoichiometry of the studied reaction between crystal violet and NaOH. During the course of the reaction, the reaction mixture color becomes less and less intense, ultimately becoming colorless when all the crystal violet has been consumed [9].

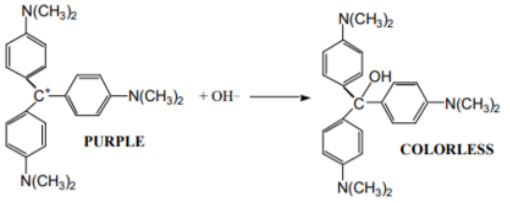


Figure 7 - Reaction between crystal violet and NaOH used to study mixing efficiency of the biocatalytic system (adapted from [9])

The results for the reactor mixing efficiency ultimately show that the reactor's volume affected the reaction time, since the results are different for the 3 volumes (figure 8). At the 40mL reactor, the mixing time probably was somewhat higher than 360 seconds, because the curves for the color channels did not reached a plateau for 360 seconds, but presumably after that. Table 8 clearly shows that the naked-eye and inflexion point do not provide the same results in terms of mixing time. Overall, these results could be a possible explanation for the difference in activity for the hydrolysis of *p*-NPB at different reactor volumes. A magnetic agitation that increases with an increase in the reaction volume, could provide a reduction in the time required for the mixtures to become transparent faster, which is the desired time to achieve a necessary mixing and homogeneity through the reactor.

Table 7 – Reactors mixing time and relative mixing efficiency, where the 10 mL reactor is the reference

Scale (mL)	Mixing Time (sec)	Relative Mixing relative efficiency (%) Reference: 10 mL scale
10	260	100 %
20	300	84.61 %
30	360	61.54 %

Table 8 – Mixing time determined by naked eye and image analysis

Scale (mL)	Mixing time (sec) – naked eye	Mixing time (sec) – inflexion point
10	240	280
20	300	320
40	360	>360

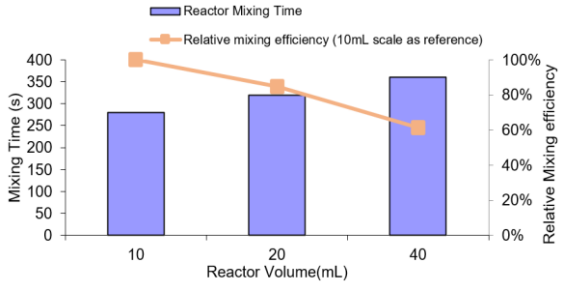


Figure 8- Reactors mixing time and relative mixing efficiency, where the 10 mL scale is the reference

3.3. Interfacial area of a biphasic system as measure of biocatalytic activity for different reactor volumes

The aim of the study was to verify if the interfacial area was the same across the reactor volumes, given the fact the previous results, regarding reactors mixing efficiency, showed that there were differences across the scales. The interfacial area is an important parameter that defines mixing efficiency in aqueous-organic systems. A large interfacial area increases the rate of mass transfer[10]. An aqueous-organic biphasic reaction system possesses several advantages for biocatalytic reactions. It has minimum requirements for mixing and beneficial conversion of poorly water-soluble substrates and its advantageous when using in *situ* product removal for poorly water-soluble products. Using and aqueous-organic biphasic also holds some disadvantages such as a potential mass-transfer limitation, potential emulsification of phases which makes separation difficult and potential interfacial denaturation of the biocatalysts[10].

The biphasic aqueous-organic system used for this experiment was composed of an aqueous and an organic phase. The system was composed by the enzyme EH, Tris-HCl, MPP and hexane. Sampling was done at two time points for each reactor volume, and the samples were loaded into a concave lamella to preserve the integrity of the substrate bubbles. The first one at stationary state and the second to half of the conversion where supposedly substrate bubbles are smaller, corresponding to 25 and 50% of substrate conversion, respectively. To obtain the number of pixels and the μm^2 area, 2 measures were needed: The Horizontal Field Width (HFW) and the Vertical Field Width (VFW) (Table 9)

Table 9 – Horizontal Field Width and Vertical Field Width of the processed images in pixels and mm

Units	HFW	VFW
Pixels (10 x 150 magnification)	2558	1918
µm (10 x 150 magnification)	800	600

Figures 9 and 10 represent the bubble radius frequency for a range of substrate bubbles radius across

the reactors. Looking at Figure 10, the image related to the bubble radius frequency across the scales when 25% of substrate conversion is obtained, it was detected that bubbles with a radius ≤ 5 μm are in greater number at the smallest scale(10 mL), but when the bubbles had a substrate radius between 5 and 10 μm, their frequency was higher at the biggest scale(40 mL). The results at 50% of substrate conversion suggested that the scale which had the most bubbles with a radius ≤ of 5 μm was the 40 mL and when the bubble radius was between 5 and 10 μm, the scale which had the most bubbles was the smallest. This represented a change in the frequency of bubble size radius distribution across the scales. Regarding the middle scale (20 mL), it followed an expected pattern in the distribution of the substrate bubble radius frequency at 25% of substrate conversion, but when looking at the same distribution at 50% of substrate conversion, the results show a relatively high reduction of the substrate bubble radius frequency, mainly at the lower radius.

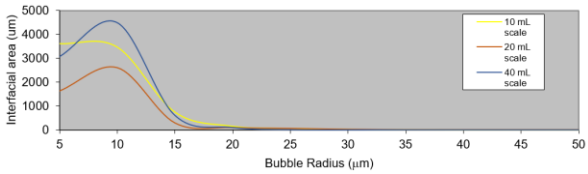


Figure 9 – Bubble radius frequency across the scales when a 25% of substrate conversion was reached

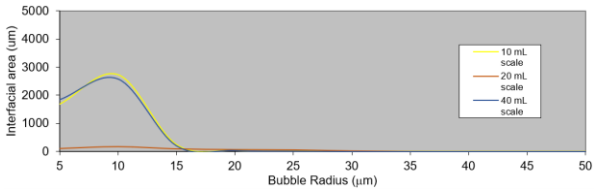


Figure 10 – Bubble radius frequency across the scales when a 50% of substrate conversion was reached

Figure 11 is the representation of the Interfacial area when both 25% and 50% of substrate conversion were reached. When looking at both the figure 11 and Table 10, it could be quickly understood that there was a great similarity of interfacial areas between the 10 and 40 mL reactors and at the same time a great disparity comparing both scales to the 20 mL reactor, especially when 50% of substrate conversion was reached.

Table 10 – Interfacial area across the reactors

	Area (μm²)		
	25 % substrate conversion	50 % substrate conversion	Total
10 mL reactor	440199,96 μm²	254123,43 μm²	694323,39 μm²
20 mL reactor	267223,87 μm²	43730,97 μm²	310954,84 μm²
40 mL reactor	448839,34 μm²	248405,73 μm²	697245,07 μm²

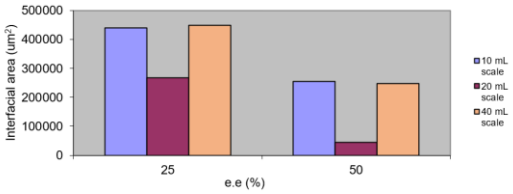


Figure 11 – Interfacial area across the scales when 25% and 50% substrate conversion were reached

This experiment had the goal of obtaining the interfacial area for different reactor volumes, to understand if the mass transfer, when specific amounts of substrate conversions were attained during the reaction course. The biggest number of substrate bubbles, at all the reactor volumes at 25 and 50% of substrate conversion were found to have a radius comprised between 5-10 mm, meaning that the smallest bubbles were responsible for most of the interfacial area, which is, in fact, a meaningful result, because smaller bubbles lead to more interfacial area, and consequently to a better mixing efficiency.

In a comparative study[11] that used a capillary-microreactor to analyze the nitration of a single ring aromatic in an exoteric liquid-liquid two-phase reaction, where mass transfer experiments with different flow velocities, but identical residence times, yielded differences in conversions and amounts of by-products formed. When the flow velocity is increased, the conversion rate and the amount of by-products also increase. Increasing velocity also results in enhanced mass transfer, since the intensified interphase transport, not only increases the mass transfer of the reactant into the reaction phase, but also increases the extraction of the products and intermediates. Another study[12] also confirms that the increase in linear velocity, increases the mass transfer coefficients, due to increase in the interfacial area. In this study, the agitation was constant at all reactor volumes, and apparently sufficient for a good mass transfer at the 10 and 40 mL reactors.

Looking at the direct comparison of the interfacial area between the scales, its clear that the 20 mL reactor had distinct results comparing to the other scales. The results showed a smaller interfacial area for the 20 mL, but not for the 40 mL scale. Normally, Bigger interfacial will lead to better distribution and a higher activity in a biphasic system[13]. When the interfacial area increases, the substrate concentration in the aqueous phase decreases, because the substrate concentration in the organic phase increases. Even though the substrate concentration of the organic phase depends on the physicochemical characteristics of the organic phase and the substrate could be directly transported and used by cells adhering to the interfacial area[14]. Following studies of biphasic systems mixing efficiency during scale-up, should consider using a live analysis mechanism to evaluate the evolution of the interfacial area during the whole reaction time, and not just at some time-specific points, to provide more robust

results, since this experiment was performed with a series of 24 photos for each sample in duplicate.

3.4. Substrate concentration and reactor volume on the enantioselectivity of EH towards methyl-2-phenylpropanoate

This study aimed to evaluate the influence of 2 conditions on the enantioselective of EH. The concentration of a chiral substrate, methyl-2-phenylpropanoate, either being 40, 80 or 160 mM, and influence of the scale of the reaction. Chiral selective enzymes like EH reduce raw material costs in the synthesis of pure chiral compounds, meaning that they should be able to cleave preferentially only one chiral ester bond in a racemic mixture. Previous studies have focused on the link between the selective character of some esterases and their narrow or broad substrate spectrum. In fact, some authors that are part of the INMARE project [15] investigated the relationship between the substrate promiscuity level and chiral selectivity of a large set of 145 phylogenetically and environmental diverse microbial esterases, whose specific activity against 96 different esters, with the goal of deepen the understanding of the relationships between substrate promiscuity and chiral selectivity. This study extends the understanding of the enantioselectivity of one specific EH towards one specific racemic mixture, methyl-2-phenylpropanoate, which is a substrate that has only one chiral point, which means that is not easily catalyzed by any enzyme. In this experiment, the enzyme concentration was constant, just like in the experiment of the interfacial area, to maintain the same reaction conditions and understand if the differences in the interfacial areas with different reactor volumes are comparable to the enantioselectivity of the enzyme towards MPP.

The enantioselectivity study in an aqueous-organic system was carried by measuring the concentration of each enantiomers for different substrate concentrations, to understand the influence on the biocatalyst performance. Previous studies[16] have also studied the effects of changing the concentration of the aforementioned substrate on the enantioselectivity of enzymes, but not at different reactor volumes. EH activity towards the two enantiomers, substrate enantiomeric excess and conversion rate of the substrate are presented in table 11. The following figures 12, 13, 14, 15 and 16 relate to the enzymatic activity towards different substrate concentrations (40,80 and 160mM) at different reactors (10,20 and 40mL).

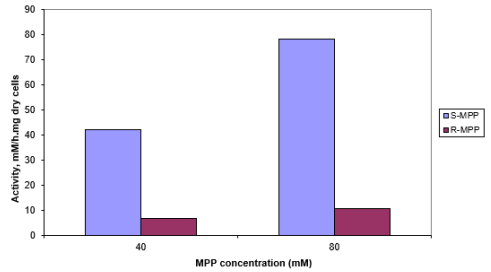


Figure 12 – Enantioselective hydrolysis of methyl-2-phenylpropionate (40mM) with lyophilized EH cells during 5 hours of reaction at the 10 mL reactor

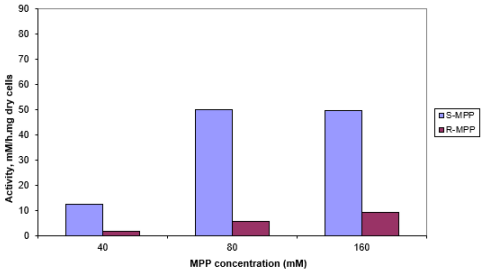


Figure 13 – Enantioselective hydrolysis of methyl-2-phenylpropanoate (40mM) with lyophilized EH cells during 5h (40mM) and 6h (80 and 160 mM) of reaction at the 20 mL reactor.

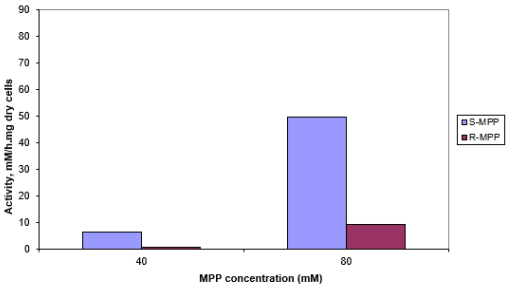


Figure 14 - Enantioselective hydrolysis of methyl-2-phenylpropanoate (40Mm) with lyophilized EH cells during 5h (mM) and 6h (80 mM) of reaction at the 40 mL reactor.

Figures 15 and 16 are the direct comparison of the enantioselectivity towards the two enantiomers of the substrate, for different reactors volumes, at 40 and 80 mM of methyl-2-phenylpropionate.

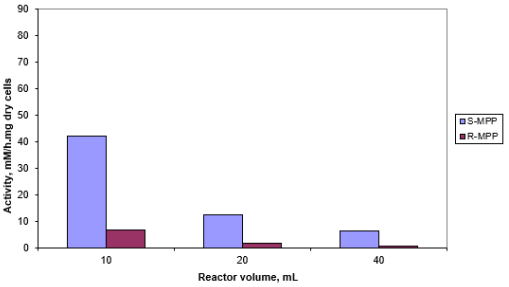


Figure 15 - Enantioselective hydrolysis of methyl-2-phenylpropanoate (40Mm) with lyophilized EH cells during 5 hours of reaction for all reactor volumes.

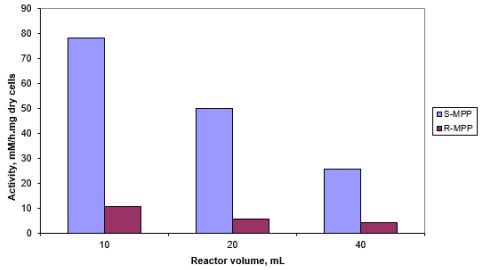


Figure 16 - Enantioselective hydrolysis of methyl-2-phenylpropanoate (80Mm) with lyophilized EH cells during 6 hours of reaction for all reactor volumes.

Table 11 - Results of the enantioselective hydrolysis of methyl-2-phelylpropanoate with the lyophilized EH cells.

Reactor Volume	[MPP], mM	Activity towards S-MPP (mM/h. mg dry cells)	Activity towards R-MPP (mM/h. mg dry cells)	E.E (%)	% Converted substrate
10	40	8.82	1.40	77.30	63.77
	80	16.44	2.23	49.01	31.48
20	40	5.27	0.67	79.56	38.61
	80	20.95	2.35	65.60	56.48
	160	20.83	3.83	33.47	26.09
40	40	21.46	3.38	78.19	44.76
	80	5.40	0.54	56.19	55.20

According to the results, the activity of the enzyme towards both enantiomers, S-MPP and R-MPP increased with the increase of the substrate concentration for all reactor volumes, and this may indicate that for lower substrate concentration, the enzyme was used in excess. The highest enzymatic activity towards the S-MPP was attained in the 10 mL reactor volume with a substrate concentration of 80mM. In terms of enantiomeric excess, when the substrate concentration was increased from 40 to 80 and 160 mM, it was verified that the e.e decreased in all reactor volumes, although in was relatively maintained between 77-80% for the reaction with 40 mM of MPP at all reactor volumes. These results may be the consequence of a decrease in the reactor mixing efficiency when the reactor volume is increased. These results can be compared with the reactor mixing efficiency study, where the times at which the inflexion point of the RGB channels were attained, increased with the increase in the reactor volume. Regarding the effect of the increase of the reactor volume in the activity of the enzyme towards both enantiomers, it was observed that the activity decreased with the increased of the reactor volume, just like the enantiomeric excess, and this can also be correlated to the mixing efficiency study.

Overall, when observing the results from the increase in the reactor volume in table 10, it can be concluded that the used esterase was enantioselective for all substrate concentrations, presenting higher enantiomeric excess for the lower substrate concentration, and higher activity rates for higher substrate concentrations. The obtained results and previous ones [16], when compared to the literature, are lower and so are not suitable for enzymatic resolution processes that require high enzymatic yield and high enantiomeric excess. It should also be mentioned that further studies should focus on the extent that the selectivity of this enzyme changes towards the enantiomers in different reaction conditions. The uniqueness of this work relies on the tentative of the correlation between the reaction mixing efficiency and the interfacial area with the enantioselectivity of the enzyme, in order to understand the influence that parameters like mass transfer have on the activity of an enzyme when increasing the reactor volume.

4. Conclusion and future work

Regarding the study that compared how many freezing and thawing cycles could the lyophilized and wet paste cells resist, it was found that lyophilized cells conserve better their catalytic activity in comparison to wet cell paste, possibly to the fact that wet cell paste are most prone to bacterial contaminations than lyophilized cells, even considering that eventually after some hours at a storage temperature of +4ºC, both enzyme samples would be contaminated for example by proteases.

The enantioselectivity of EH towards methyl-2-phenylpropanoate at different reactor volumes was assessed by enzyme's conversion rate for both enantiomers, as well as the enantiomeric excess through a 5h reaction for a lower substrate concentration (40 mM) and 6h reaction for a higher substrate concentration (80 and 160 mM). The reactors mixing efficiency and the interfacial area were the evaluated parameters that influenced the mass transfer and consequently the maintenance of the activity when the reactor volume was increased.

In the study that aimed to evaluate the effects of 2 parameters, reaction temperature, and light, on the activity of Abo and EH towards the substrate *p*-NPB, it was expected that a higher temperature under dark conditions, could mitigate the differences in the enzyme's activity found for different reactor volumes. EH was found to be the most stable enzyme during reactor volume increase at 30ºC under dark conditions, but when the reaction system was, at 22ºC and light conditions, it showed higher activity when comparing to the previous conditions (30ºC, dark). Regarding Abo activity, when increasing the reactor volume, the difference between reactor volumes was bigger at 30ºC under dark conditions. The differences in the biocatalyst's activity on such a small reactor volume increase was the motivation for the reactors mixing efficiency study and the study related to the interfacial area.

The previous results on the activity of Abo and EH for different reactor volumes, indicated that there was a possible mixing efficiency problem since there was a loss in catalytic activity when the reaction volume was increased. The mixing efficiency study evaluated the time that was needed for the reaction mixture, with a tracer (crystal violet), needed to become transparent. The results given by "Fiji" (image processing software)

showed that there was a considerable decrease in the mixing efficiency, that could be explained by the increase in the mixing time needed when the reaction volume is increased. An increase in the magnetic agitation that follows the increase in the reaction volume, could provide a reduction in the time required to obtain a defined degree of uniformity, which relates to the desired time to achieve a necessary mixing and homogeneity through the reactor. An increase in the magnetic stirring bar size could also be a possible solution, since the increase in the mixing bar diameter, results in larger circulatory velocity, which causes a theoretical reduction of the mixing values. Further work would require a more extensive evaluation of the most important observable parameters present in Figure 19.

Another study that intended to evaluate the mass transfer in the aqueous-organic system was the study of the interfacial area during the enantioselective hydrolysis of MPP. The interfacial area comparison between the scales showed that in the 20 mL reactor, the system had distinct results comparing to the other reactor volumes. The expected results would be a continuous drop in the interfacial area as the reactor volume increases, which happened at the 20 mL reactor but not at the 40 mL reactor, which means that the interfacial area for the 40 mL reactor was not a limiting factor. According to the literature, a bigger interfacial area leads to better distribution and higher activity in a biphasic system, because as the interfacial area increases the substrate concentration in the aqueous phase decreases. Following studies on biphasic systems mixing efficiency during scale-up, should consider using a live analysis mechanism to evaluate the evolution of the interfacial area during the whole reaction time, to provide more robust results, since the experiment was performed by extracting a series of 24 photos from a video, that were related to some specific time points.

These previous studies had the goal to provide explanations and further indications for the scale-up effect on the enantioselectivity of EH towards methyl-2-phenylpropanoate. Overall, the results obtained during reactor volume increase showed that EH cells were enantioselective at 40, 80, and 160 mM substrate concentration, presenting higher enantiomeric excess for the lowest substrate concentration and higher conversion rates for the highest substrate concentration. For an industrial process scale-up, this work cannot be directly applied, due to the lack of maintenance of substrate conversion rate across the scales, but it can be valuable to understand the enantioselective of the lyophilized EH cells towards MPP, and also understanding the underlying factors behind the low conversion rates at small scales. If the strategy to scale up, was to define a conversion rate as a reaction parameter, there would need to be an increase in the substrate that was supplied to the reactor, which would not be economically feasible, and would probably lead to substrate inhibition and less enantiomeric excess. So, for further work, there would need to be a better understanding of the reaction environment for this enantioselective reaction.

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