TÉCNICO LISBOA

Stability and enantioselectivity of selected marine enzymes

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Thesis to obtain the Master of Science Degree in

Biological Engineering

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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 September 2019-September 2020, under the supervision of Prof. Carla da Conceição Caramujo Rocha de Carvalho. The thesis was co-supervised by Dr. Rainhard Koch

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 start by thanking Professor Carla Carvalho for the opportunity to develop my master thesis alongside with Dr. Rainhard Koch.

To professor Carla, I would like to express gratitude for all the knowledge that was transmitted to me. I deeply appreciate what I've learned about bioprocess engineering, biocatalysis, and other related topics, so once again, thank you, Professor Carla.

I would also like to express my gratitude to Dr. Filipe Carvalho, that helped me a lot in the development of my experiments, especially because of the knowledge he provided me on biocatalysis.

To everyone from the IBB-BERG, that one way or another helped me to complete this work, know that I appreciate your time and dedication to help newcomers.

Last but not least, I need to express how grateful I am to have such a supportive and interested family and friends, especially my parents and my brother Henrique, that have been aiding me in all my academic and personal projects.

This document was written and made publically available as a legal academic requirement and as a part of the evaluation of the MSc thesis in Biological Engineering of the author at Instituto Superior Técnico. The work desctibed herein was performed at iBB-Institute for Bioengineering and Biosciences of Instituto Superior Técnico, Universidade de Lisboa, during the period of September 2019-September 2020, under the supervision of Prof. Carla da Conceição Caramujo Rocha de Carvalho. The thesis was co-supervised by Dr. Rainhard Koch.

Resumo

A biocatálise permite a síntese de compostos de forma sustentável e poderá substituir a catálise química em vários processos. A biocatálise ou a catálise mediada por enzimas, tem diversas aplicações na indústria, como por exemplo na indústria farmacêutica. Este trabalho teve como objetivo estudar o efeito da temperatura de armazenamento na atividade das enzimas selecionadas e entender quais os fatores que influenciam a atividade e enantioselectividade das enzimas em bioreactores com diferentes volumes.

A performance catalítica de duas esterases com origem em microrganismos marinhos, com potencial aplicação industrial como biocatalisadores, foi testada em reatores com 3 volumes com o objetivo final de compreender quais eram os parâmetros mais importantes a avaliar. A resolução enantiomérica do substrato metil-2-fenilpropanoato, foi avaliada com a esterase EH num sistema orgânico: aquoso bifásico nos 3 volumes, 10,20 e 40mL.

Neste estudo verificou-se que parâmetros como o tempo de mistura e a área interfacial variam entre os três volumes de reactores. Também se verificaram diferenças na enantioselectividade das enzimas EH relativamente ao substrato metil-2-fenilpropanoato nos 3 volumes. Tanto a estabilidade como a atividade das enzimas EH e Abo relativamente ao substrato *p*-nitrofenil butirato foram avaliadas a diferentes temperaturas de armazenamento, -16ºC e +4ºC.

Várias as experiências planeadas inicialmente para este trabalho, não puderam ser realizadas devido à Covid-19.

Palavras-chave: Enzima, Biocatálise, enantioselectividade, reactor

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. The enantioselective resolution of the racemic substrate methyl-2-phenylpropanoate was performed with the enzyme esterase EH in an biphasic 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*-nitrophentl butyrate were assessed at different storage temperatures, -16^{o}C and +4^{o}C.

Several experiments initially planned for this work, could not be conducted due to COVID-19

Kewywords: Enzyme, biocatalysis, enantioselectivity, bioreactor

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Abbreviations

(*R*)-HBPA - (R)-2-hydroxy-4-phenylbutyric acid

(*R*)-HBPE - Ethyl (*R*)-2-hydroxy-4-phenylbutyrate

ACE – Angiotensin converting enzyme

ACR – Agitated cell reactor

API – Active Pharmaceutical Ingredient

CAGR – compound annual growth rate

CLEA – Crossed Linked Enzyme aggregates

CPBR – Continuous Packed bed reactor

CPM - Continuous Pharmaceutical manufacturing

CSTR – Continuous stirred tank reactor

Da - Damköler number

DO – Dissolves oxygen

DoE – Design of experiments

dsDNA – double-stranded DNA

EH – Ester hydrolase

E. coli – *Escherichia coli*

e.e. – enantiomeric excess

ES – Enzyme-Substrate Complex

FB-STR – Fed-batch stirred tank reactor

GC – Gas chromatography

HFW – Horizontal Field Width

HGP – Highest growth potential

HPLC – High pressure liquid chromatography

ISPR – *in situ*-product recovery

PAC - phenylacetylcarbinol

MPP - methyl-2-phenylpropanoate

P/V – Volumetric Power Consumption

PAL - phenylalanine ammonia lyases

PCMC – Protein-coated microcrystals

PFR - plug flow reactors

p-NP - *p*-nitrophenol

p-NPB - 4-Nitrophenyl butyrate

RGB – Red Green Blue

STR – Stirred tank reactor

VFW – Vertical Field Width

I. Objectives and Overview

Enzymes are powerful biological catalysts produced by living organisms that increase the rate of biochemical reactions, at moderate temperatures. Enzymes are widely used in industry-scale catalysis, pharmaceutical research, and development, mainly due to their high product selectivity, operation under mild condition and low environmental impact when compared to chemical reactions. Biocatalysis can be performed by using both isolated enzymes and whole cells as catalysts in an aqueous: organic biphasic system. Enzymes inside cells are in a protected environment and have often more stability than when isolated. However, whole cells may have lower reaction rates than isolated enzymes because the substrate needs to be able to transpose the cell envelope to reach the enzyme(s). The majority of recent studies published on biocatalysis with whole cells used organic or organic: aqueous media, in this work, it was used an organic: aqueous media[1].

Biocatalysts have some advantages over chemical catalysts, regarding the reduction of the total number of processing steps, and according to some published reports, biocatalysts have been used as an important tool for the production of some chiral compounds, meaning that there has been an increasing interest in developing more eco-friendly processes, alternative to conventional chemistry [2][3][4]. Biocatalysis has already been exploited by pharmaceutical companies to manufacture some active pharmaceutical ingredients (API's) with success, which resulted in a great number of pharmaceuticals available on the market with intermediates produced by biocatalysts. Some recent API's commercially used are amoxicillin, pregabalin, sitagliptin, and many others [5].

Biocatalysis is becoming a very important asset for pharmaceutical industries, that must be in harmony with chemical processes, having the objective of providing "greener" alternative solutions with reduced environmental impact. The interest in the development and application at an industrial scale has also been increasing, but there is still a lack of standardized protocols and collaborations between chemists and engineers for biology-related areas, that would increase scale-up implementation of lab-scale biocatalytic processes [6] [7].

One of the main challenges in industrial biocatalysis is to scale-up a biological reaction from the laboratory to an industrial process. A successful scale-up of a biocatalytic process requires a profound understanding of the interactions between the biocatalyst and the physical and chemical environment in the reactor. Therefore, both reactor selection and operation conditions are important because one can control the reaction environment across the scales, so that the predictions made as the scale changes are accurate [8].

Having in mind the current interest in understanding and developing new scale-up strategies in bioprocesses, more specifically in biocatalytic processes that aim to give as an output high enantiopure products, the objectives of the master thesis were:

i. to study the effect of storage temperature on enzyme stability;
ii. to study parameters that affect the activity and enantioselectivity of enzymes in bioreactors with different volumes at a small scale.

The enantiomeric selective hydrolysis of a racemic mixture of methyl-2-phenylpropanoate was used as model reaction.

1. General Introduction

1.1. Marine Biotechnology

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 hydrolyzing 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].

1.2. Biocatalysis

1.2.1. Historical and recent developments

Rene Reaumur and Lazzaro Spallanzani provided knowledge on gastric digestion of meat, but it was Reaumur's experiments in the 18th century related to the digestion of meat that are considered the first studies of the enzyme's activity[17]. In parallel with these works, fermentation developments and starch hydrolysis also contributed for the early growth of enzymology. It was Gottlieb Kirchhoff who discovered α-amylase activity when he was characterizing the chemical hydrolysis of starch to sugar, in 1815 [18]. His work was followed by Anselme Payeb and Jean Persoz, who were responsible for the first attempts of enzyme purification when they enriched the hydrolytic activity of malt gluten calling it diastase (1833) [19].

In 1877, Wilhelm Kühne, a German physiologist, was the first to use the word "enzyme", when he described the capacity of yeast for the production of alcohol from sugars [20]. A French scientist called Pierre Duclaux and Gabriel Bertrand, used the term diastase, relating to what is called today as enzymes. The suffix "ase" was recommended for all enzyme names (1889) [21]. It was years later that James Sumner provided an important work on the understanding of the protein nature of enzymes when he found that urease was a protein that could be isolated and crystallized (1923) [22]. At the end of the nineteenth century, the number of enzymes reported became so large that in 1955 enzymes were organized into categories. That was when the International commission of Enzymes was founded to systematize the classification and naming of enzymes.

In the 1980s it was discovered that some RNA molecules called ribozymes were also able to exert catalytic activity [23]. In the same decade, some biochemists developed the technology to generate antibodies with catalytic properties [24]. Later in 1997 Nobel prize award winners, Paul D. Boyer and John E. Walker discovered how enzyme ATP synthase catalyzes the formation of ATP and Jens C. Skou discovered the first ion-transporting enzyme, Na+, K+ -ATPase [25][26]. Other notable works, such as the study of the transition states of chemical reactions using femtosecond spectroscopy, by A. Zewail (1999) and the discovery of ubiquitin-mediated protein degradation by A. Ciechanover, A. Hershko, and I. Rose, were also rewarded with the attribution of the Nobel prize in chemistry [27][28].

From 2000 onwards, biocatalysis research speeded up, mainly due to the advances in metagenomic sequencing and protein engineering, and according to J. M. Woodley, several hundred of processes are now in operation, particularly in the pharmaceutical industry[6]. Advances in protein engineering and directed evolution techniques over the last two decades, have allowed enzymes to adapt to the harsh conditions of industrial processes with high temperatures and the presence of organic solvents. The discovery of enzymes from organisms that live in extreme environments has channeled for industrial applications. For example, some glucoamylases and amylases perform very well in the hydrolysis of starch at temperatures above 100ºC, and some enzymes, such as lipases, show stability in highly hydrophobic solvents[29].

1.2.2. Biocatalysts versus synthetic catalysts

The following table 1 gives information about the advantages and disadvantages of biocatalysts and the differences between biological and synthetic catalysts.

Table 1 – Common advantages and disadvantages of biocatalysis found in the literature[8][30][31][32][33][34][35][73]

Advantages	**Disadvantages**
• Exceptional substrate selectivity • High catalytic efficiency • High chemo- regio- enantiomeric selectivity • Lower production cost compared to chemical catalysis • Mild reaction conditions • Low environmental and physiological toxicity • Less waste production than chemical processes • Produced from inexpensive renewable resources • Multi-step reactions in single strain with cofactor regeneration • Simplified downstream processing • Can be reused (immobilization techniques)	• Lack of enzyme stability at extreme temperatures/pH and on turbulent flow regimes • Often not sufficiently stable in the desired media • Susceptible to substrate or product inhibition • Undesirable metabolic by-products • Many enzymes are cofactor-dependent, high costs associated • Many Biocatalysts in their native form, present a low substrate scope

1.3.3. Enzymes: Basic Concepts and Kinetics

Enzymes classification is based on the type of reactions that they catalyze (Table 2).

Table 2 - Enzymes international classification (adapted from [37])

No.	Major Class	Type of reaction catalyzed	Enzymes
EC1	Oxidoreductases	Transfer of hydrogen/oxygen/electrons between molecules	Dehydrogenases, oxidases, oxygenases, peroxidases
EC2	Transferases	Transfer of groups of atoms from one molecule to another	Fructosyltransferases, transketolases, acyltransferases, transaminases
EC3	Hydrolases	Hydrolytic cleavage of bonds	Proteases, amylases, acylases, lipases, phosphatases, cutinases
EC4	Lyases or Desmolases	Non-hydrolytic cleavage by elimination or addition reactions	Pectate lyases, hydratases, dehydratases, decarboxylases, fumarase, argino succinase
EC5	Isomerases	Transfer of group from one position to another within one molecule	Isomerases, epimerases, racemases
EC6	Ligases or synthetases	The covalent joining of two molecules coupled with the hydrolysis of an energy-rich bond in ATP or similar triphosphates	Synthases, ligases
EC7	Translocases	Catalyze the movement of ions or molecules across membranes or their separation within membranes	Ubiquinol oxidase

An enzyme typically catalyzes a single chemical reaction or tightly related reactions, while side reactions responsible for the formation of by-products, are uncommon in enzyme-catalyzed reactions. Many enzymes consist merely of protein (apoenzymes), but many as well contain cofactors (holoenzymes), that are important for the catalytic activity of an enzyme. Cofactors can be divided in two groups, small organic molecules (coenzyme), and metals. Coenzymes can be tightly bound, and when that happens, they are generally called the prosthetic group of enzymes.

The simplest Michaelis-Menten model for enzyme kinetics is where the enzyme has only two conformational states E and ES. In most in vitro experiments with single enzymes, the concentrations of S and P are approximately constant. The establishment of an enzyme-substrate (ES) complex is the first step in enzymatic catalysis, where the substrate binds to the active site of the enzymes. The evidence for the existence of the ES complex is explained by figure 1 where it is observable that when the substrate concentration is increased, the reaction rate increases until the maximal velocity is reached.

$$E + S \rightleftharpoons ES \rightharpoonup E + P \tag{1}$$

The Michaelis-Menten equation for this system is:

$$V_0 = \frac{V_{max}[S]}{K_M + [S]} \tag{2}$$

Where V_{max} represents the maximal velocity achieved by the system at maximum (saturating substrate

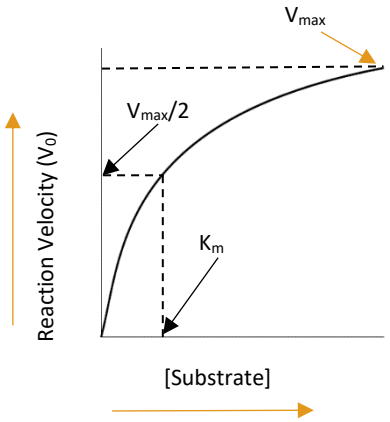


Figure 1 - Reaction velocity versus substrate concentration in an enzyme-catalyzed reaction. Maximal velocity (Vmax) is approached when practically all the catalytic sites are filled, or saturated, given that, the reaction rate cannot increase. The Michaelis-Menten (K_m) is the substrate concentration yielding a velocity ($V_{max}/2$) (adapted from [56] [57]).

concentrations. K_m is the substrate concentration at which the reaction velocity is 50% of the $V_{max.}$ [S] is the concentration of the substrate S. Figure 1 is a plot of the Michaelis-Menten equation's predicted reaction velocity as a function of substrate concentration, with the significance of the kinetic parameters V_{max} and K_m graphically described.

One of the first steps in a enzymatic kinetics study is to determine the product formation or substrate consumption as a function of time, for several substrate concentrations, without changing enzyme quantities. Is projected that in each selected substrate concentration, the amount of product formed increases with time, until the active site of the enzymes is saturated, and the increase in product concentration reaches stagnation (V_{max}), this means that the reaction equilibrium has been reached. This can be better represented by the initial rate of catalysis (V_0) as the number of moles of product formed per second when the reaction time is 0, and then plotted with substrate concentration, maintaining enzyme concentration constant (Figure 2).

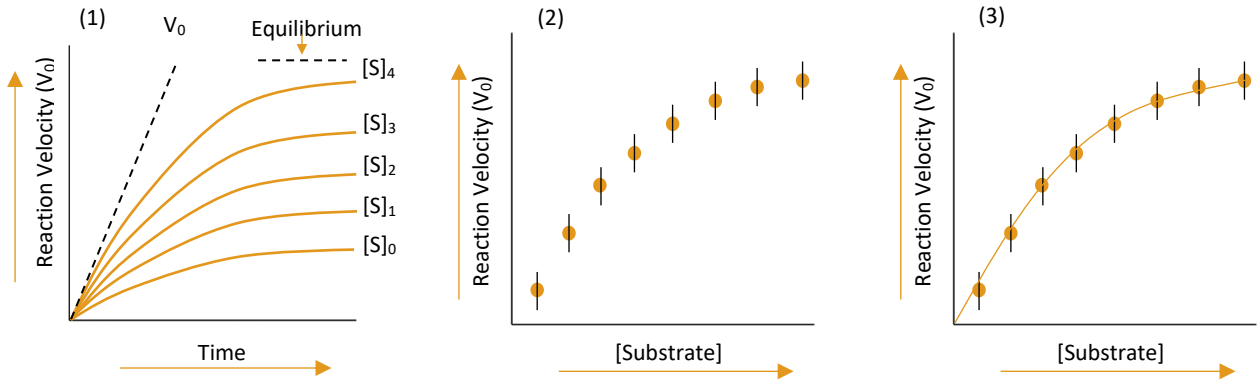


Figure 2 - Illustrated process to attain the relation between initial velocity (V_0) and substrate concentration ([substrate]). (1) Substrate consumption or product formed at different substrate concentrations plotted as a function of time. V_0 for each substrate concentration is determined from the slope of the curve at the beginning of the reaction. (2) V_0 values are plotted, with error bars, against substrate concentration. (3) Data points are connected. (Adapted from [57] [58])

As it can be observed from figure 2 the reaction velocity increases linearly as substrate concentration increases and then begins to level off, approaching a maximum at higher substrate concentrations [57] [58]. In this work, the relationship between initial velocity, V_0, and the concentration of the subtrate 4-nitrophenyl butyrate was assessed for both enzymes, Abo and EH.

1.3.4. Activity and Enantioselectivity during scale-up

Enzyme activity concept

Enzyme activity can be described as the quantification of the substrate and product concentration changes: decrease in substrate concentration or the concentration of product formed in a defined period. As it was observed from figure 1, enzyme-catalyzed reactions require less activation energy to convert substrate into product compared to uncatalyzed reactions, which increases the rate of product formation [40].

Enzyme assays are fundamental to evaluate enzyme activity, either by measuring substrate consumption or product release in time. One can choose between continuous assays or discontinuous assays, where the objective is to obtain a continuous reading of activity or to read the activity of samples in specific periods [41]. In this work, the spectrophotometer was used to evaluate enzymes catalytic activity in both continuous and discontinuous assays.

Reaction Parameters

An enzyme-catalyzed reaction is influenced by reaction parameters, like temperature, pH, and E/S ratio. When the reaction temperature increases, so does the reaction rate. There is a specific temperature at which an enzyme's activity is maximum. Above that temperature, the enzyme keeps increasing its kinetic energy to a point that its structural organization starts to collapse (Figure 3 - (A)).

In terms of pH sensitivity (Figure 3 - (B)), enzymes are quite specific, because they work at their prime in a small pH range. The optimal pH, where the enzyme is most active, does not follow a standard, meaning that each enzyme has its optimal pH range. Above or below it, enzymes start to lose their activity, because pH can either promote or break molecular bonds, altering the enzyme's conformation [40][42][43].

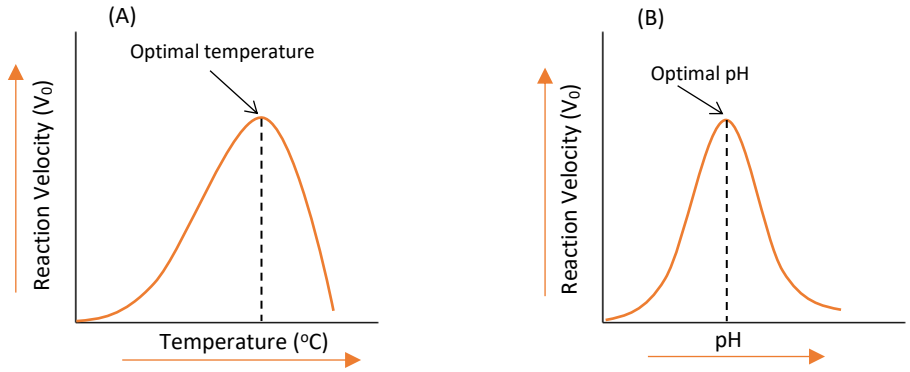


Figure 3 - (A) Effects of Temperature on the reaction rate. (B) Effects of pH on the reaction rate.

Enantioselectivity

Stereoisomers have different spatial configurations (spatial atoms configuration) and normally the same chemical and physical properties. There are different classes of stereoisomers, but the ones that have an interest in this work are enantiomers and diastereoisomers.

Enantiomers are chiral molecules, that are non-superimposable mirror images of each other. They have identical physical and chemical properties such as identical melting points, pKa, solubilities, but the difference between them is that each one rotates the plane of polarized light to the same degree but in opposite directions. Given that, the magnitudes of the bond lengths, angles, and torsional angles are identical for both enantiomers, it can be assumed that both have identical activation energies (E_a) and free energy.

Diastereoisomers are any pair of stereoisomers (isomers of the identical constitution but different three-dimensional architecture) that are not enantiomers. A pair of diastereoisomers have different molecular shapes and volumes, and so, they will possess distinct physical properties, such as melting point and solubility (Figure 4)[44][95] [96].

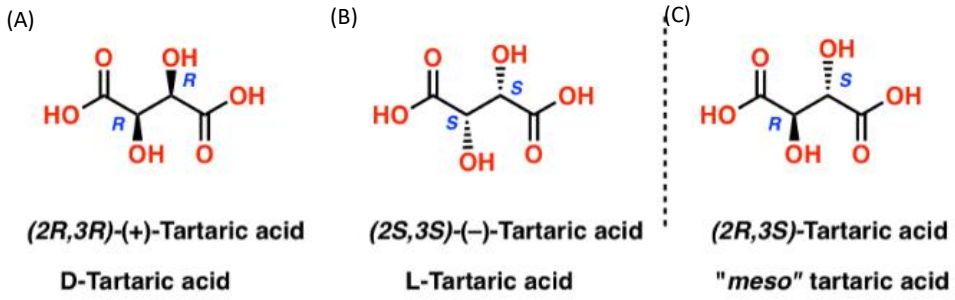


Figure 4 – Three stereoisomers of tartaric acid (2,3-dihydroxysuccinic acid) A and B are enantiomers, A and C, B and C are diastereomers)

The enantiomeric excess or *ee%* is a way to measure the enantiomer composition of a sample that describes the excess of one enantiomer over the racemate, which can be calculated by the equation:

$$ee\% = \frac{[S] - [R]}{[S] + [R]} \times 100\% \qquad 3$$

The term enantiomerically pure or optically pure molecule is applied when the chiral molecule only contains one enantiomeric form. The most used methods to analyze and determine the enantiomeric composition of a chiral compound are chromatography with chiral stationary columns, such as chiral gas chromatography or chiral high-pressure liquid chromatography.

Scale-up of enzymatic reactions

Scale-up of all processes, either being biocatalytic or not, are often associated with reduced process performance. The scale-up of enzymatic processes is hampered by limitations in biocatalyst stability and mass transfer. Enzymes from marine microorganisms, like marine extremozymes, due to their resistance to temperature, pH, salt, and pollutants, are promising biocatalysts for new and sustainable industrial processes, that epitomize opportunity for biotechnologically application[6]. This means that there is an increasing interest to understand marine microorganisms that encode promiscuous or non-promiscuous enzymes, higher or lower levels of chiral selectivity, solvent-resistant or solvent-active and stable, and low- of high-pH/temperature-active[47]. More understanding of marine enzymes will lead to new bio-catalyst solutions in several industrial sectors.

In comparison with the development of a conventional chemical process, bioprocess design problems are frequently challenging due to the lack of precedent and, to some extent, the additional complexity from intra-process interactions. Consequently, there is a particular need for a systematic design framework which can handle a large range of different design problems and guide the engineer through the whole bioprocess development. Although significant progress has been made, such a framework has yet to be developed and here we suggest some useful engineering tools to assist in bioprocess development to facilitate knowledge-based decision-making.

The scaling-up process of enzymatic reactions from lab scale to industrial scale, faces several problems, which result in a lower enzymatic activity and enantioselectivity. Immobilized biocatalysts for example, have improved stability in broader range of operating conditions, like pH and temperature, and many other characteristics that are interesting for an implementation in an industrial scale process. The problem that urges is that the immobilization must provide a lot of improvements over the free enzyme catalyzed processes, otherwise is not economically feasible, because immobilization techniques like carrier-bond attachment, encapsulation and crosslinked enzyme aggregates are expensive. One way that was found to improve profitability of immobilized biocatalytic processes was by applying protein design via directed evolution, allowing to improve activity, stability, and substrate affinity and at the same time, reduce costs associated. Researchers at Codexis® and Merck® have in fact designed an economically competitive enzyme-catalyzed process to produce a pharmaceutical used for diabetes (subtilisin), using multiple iterations to increase enzyme-substrate affinity. Nonetheless, these advances in protein engineering are not enough to increase the number of applications in industrial enzyme catalysis, because some limitations keep unaddressed, such as the poor mechanical stability, limited reusability of the biocatalyst and the costs associated with their production in vitro[30].

Some problems that often urge during scale-up processes include:

- Biocatalysts are often perceived as too expensive to bring about an economically feasible process
- Development of an optimized biocatalytic process takes a long time and requires different competencies
- Is difficult to estimate the probability of success
- Difficult to evaluate the cost of different biocatalytic processes for lack of data on the factors that contribute to the total cost

Requirements for Scalable Industrial Biocatalytic Processes

One of the most important factors for a successful scale up of a biocatalyst process is economic requirements. In economic requirements for any new biocatalytic process, not only the potential manufacturing cost of the product needs to be considered, but also the costs of the biocatalyst as the costs associated to the process development. The feasibility of the process is usually more about evaluating the cost and time of the development rather than the manufacturing cost. The following parameters influence the economic feasibility of the process[48]:

- Reaction yield – The value of the product should exceed the value of the substrate and other raw materials.
- Biocatalyst yield – Measurement of the mass of product obtained or substrate converted by mass of biocatalyst supplied overt the operational lifetime of the biocatalyst.
- Product concentration – It's a very important parameter because it determines the scale of the downstream process, which will be sized in accordance to the recovery of a given mass of product per working volume.
- Space-Time yield – Gives information about the required plant capacity for a demand of a given product. Calculations of space-time yield will give a target for specific activity, determining how much biocatalyst is needed.

Several biocatalyst requirements should also be taken into consideration when designing a scale-up process, those are[48]:

- Physical Stability – Regardless of the biocatalyst form, it should be able to withstand the conditions inside the reactor: presence of interfaces, shear, gradients of concentration, pH and temperature.
- Biocatalyst reuse and recycle – Recycle of the biocatalyst is really important to enable effective use of the biocatalyst and achieve sufficient biocatalyst yield.
- Cofactors – Cofactors are frequently needed for an effective enzyme operation.

Scale-up and implementation of a biocatalytic process demands robustness, due to unplanned changes to reaction conditions that may occur. In that sense, several process requirements need to be assessed, such as[48]:

- Retrofit/replacement – There are three types. The most common situation is where a replacement reaction step is considered, the second is where new processes are made for new products and the last one comprises cases where the cost of the plant is already written-off and where the chemistry is highly effective and competition from biocatalysis does not come with a sufficient economic incentive that justifies its implementation.
- Product classes – The type of product and its market are important factors for the identification of the process metrics to be used.

Synthesis of chiral drugs: Enantioselective Synthesis via Enzymatic Catalysis

Enantiomeric pure compounds can be obtained by three major sources, chiral pool, asymmetric synthesis, and kinetic resolution methods. Of these sources, asymmetric synthesis and kinetic resolution are the most potent and important methods for enantiomers synthesis. Asymmetric synthesis is described as enantioselective conversion of a prochiral substrate to an optical active product using a chiral addend via asymmetric step. Lipases for example, are often used as an asymmetric catalyst in the preparation of enantiomerically pure compounds. and it has been the most powerful and commonly applied method for the preparation of chiral drugs[49].

In a chiral drug, the enantiomers can display different biological and pharmacological traits. This means that an active isomer may bind precisely to target sites, whilst an inactive isomer may bind to unplanned targets or develop an adverse binding [95]. Enantiomeric drugs display several pharmacological effects, that can be categorized by [51]:

- Both enantiomers act on the same biological target or targets, even though one has more binding affinity over the other
- Both enantiomers act on the same biological target, although they employ opposed pharmacological activities
- Both enantiomers present independent therapeutic effects due to action on distinct targets
- One or both enantiomers may present the desirable effect, or, at the same time, one of the enantiomers causes unwanted side effects.
- The inactive enantiomer can provoke the side effects of the active antipode

There has been progress in the field of asymmetric synthesis catalyzed by biocatalysts, which is resulting in more chiral active pharmaceutical ingredients. Asymmetric synthesis is the enantioselective conversion of a prochiral substrate into an optically active product. Lipases are commonly used asymmetric catalyst for the formulation of several enantiomerically pure organic compounds [52]. The following table (Table 3) gives some practical examples of lipase-catalyzed synthesis of pure enantiomers:

Table 3 - Chiral pure enantiomers synthesized by different lipase sources (adapted from[49])

Sources of lipases	**Corresponding Esters**	**Solvent**	**Product**	**ee (%)**
Candida rugosa	rac-flurbipro-fen	n-butanol and isooctane	S(+)-Flurbiprofen	93%
Candida antartica	rac-1-(2-thienyk) alkanol	Vinyl butanoate	(R)-1-(2-thienyl) butanoate	99%
Pseudomonas cepacia	2-alkanol	Isopropenyl acetate	(S)-2-alkanol	95%
Porcine pancreatic	(±)-melonol	Vinyl acetate	(R)-menolol	94%
Candida antartica	rac-sulcatol 2	Vinyl acetate	(S)-sulcatol 2 (R)-sulcatol acetate 3	>99% >99%
Candida antartica	(±)-4-methyl Pentane-2-ol	Vinyl acetate	(S)-4-Methylpentane-2-ol	≥ 99%

1.3.5. Promiscuity

Promiscuous enzymes are characterized by the ability to convert multiple substrates and are energetically more favorable in comparison to specialized enzymes, and consequently, the cell does not require many different enzymes to take up different substrates, favoring genome minimization and streamlining[53]. The active site on promiscuous enzymes can be used for its primary activity as well as for secondary activity/activities.

Enzyme promiscuity can be classified into conditional, substrate, catalytic, and product promiscuity (Table 4).

Table 4 - Enzyme promiscuity classification

Conditional promiscuity	Enzyme changes its catalytic activity according to changes in the environment. Factors responsible [54]: • Enzyme's Allosteric/Conformational alterations due to extreme temperatures, altered pH, different solvents • Protein's conformational changes by post-translational modifications • Low-affinity substrate analogs are in more concentration than the natural substrate
Substrate promiscuity	• The ability of an enzyme to metabolize different substrates • Enzymes can be engineered into a promiscuous substrate behavior [55]
Catalytic promiscuity	• The capacity of an enzyme to catalyze different reactions in addition to their native reaction(s) • Based on the principle of active site plasticity [56]
Product promiscuity	• Enzyme's ability to synthesize different products from the same substrate [57]

Substrate promiscuity is referred as the capacity of enzymes to accept a broad substrate spectrum. Promiscuous enzymes are energetically more favorable than specialized enzymes, which means that the cell does not require as many different enzymes to take up substrates. On an industrial standpoint, promiscuous enzymes are also in advantage, because those enzymes reduce the production cost of a process that normally uses multiple enzymes [58]. Another relevant enzyme's characteristics it is their chirality. Enzymes preferably, should be chiral to reduce raw materials costs in the synthesis of pure chiral compounds, which means that they should be able to cleave preferentially only one chiral ester bond, in a racemic mixture [59]. Serine ester hydrolases for example, are among the most important industrial biocatalysts, that perform important chiral reactions. Namely, the hydrolyzation of one of the chiral esters in the racemic mixtures of ibuprofen esters [60]. Several studies in fact exemplify that esters chiral preference depend on structural factors, such as the proximity of the active site. What was not known until some point, was if the selective character of this esterases, and many others is linked to a narrow or broad substrate spectrum. That was when some authors[61] investigated the relationships 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 distinct esters,

with the goal of deepen the understanding of the relationships between substrate promiscuity and chiral selectivity. The results showed that esters with low selectivity for chiral molecules where prominently esters with broad substrate spectra, whereas esterases with both high selectivity and promiscuity were nonexistent. Esterases with moderate to low promiscuity but that showed prominent selectivity was as high as 29%. This study suggests that esterases that are simultaneously promiscuous and enantioselective are rare in nature[58], and that substrate promiscuity can be a possible indicator of the esterases selectivity. Protein engineering and rational design may be an option to obtain both promiscuous and selective enzymes, that are important for industrial processes. The authors suggest that it is in fact easier to transform a promiscuous esterase into enantio-selective biocatalyst than to transform a non-promiscuous selective enzyme, because the process of increasing selectivity for an enantiomer is easier than increase the substrate spectra of a non-promiscuous enzyme[61].

1.3. Industrial Application of Enzymes

A promising collaboration between academic/research institutes and industrial partners called “INMARE” (Industrial Application of Marine Enzymes) funded by the EU Horizon 2020 Research and Innovation Programme, aimed to provide more information on the marine biodiversity in several environments and find novel enzymes that meet the need of a growing industrial enzyme market[62]. Inmare Project is defined as industrial application for marine enzymes. The project intended to find new enzymes from microorganisms that live in the ocean, in the most extreme environments, because these enzymes are already adapted to live in harsh environmental conditions, equally to industrially harsh conditions. It also aims to find promiscuous enzymes, that can accept multiple substrates, and therefore usable in more than one industrial setting.

Microorganisms have been employed for a long time by mankind to produce wine, bread, beer, and other products. It was the Babylonians and Sumerians in 6000 BC, that found a way to profit from the application of yeast to produce alcoholic beverages, without understanding the biochemical basis of their ingredients[37]. Since then, enzymes have been used in many industries. Food, agricultural, chemical, and pharmaceutical industries have been using enzyme-mediated processes for several reasons. For example, whole cells are preferably used for redox biotransformations where oxygen is typically required; chemical companies make use of biotransformations to manufacture products scale of several tons per year like the production of penicillin G/V-derived 6-aminopenicillanic acid by numerous manufacturers; high product concentrations that ease product recovery and purification [2].

Regarding their purpose of use, industrial enzymes are divided into three categories: technical enzymes, food enzymes, and feed enzymes. Technical enzymes are used in starch, textile, biofuel, pharmaceutical, and detergent companies. Food enzymes are applied to the food industry (such as dairy industry, oils, and fats industry), and the enzymes used in animal nutrition systems are known as feed enzymes [63]. Table 5 shows the major applications of enzymes in different types of industries.

Table 5 – Industrial enzymes and their major industrial applications (adapted from[64])

Type of Industry	Enzymes	Applications	Sources
Pharmaceutical Industry	Nitrile hydratase, D-amino acid oxidase, glutaric acid acylase, penicillin acylase,	Production of API’s, semi-synthetic antibiotics, biosynthetic human insulin	Bacteria, filamentous fungi, and yeast
Molecular Biological Industry	Restriction enzyme, DNA ligase, DNA polymerase	Genetic manipulation	Bacteria, hen egg white, *Thermus aquaticus*
Textile industry	a-Amylase, cellulase, pectinase, catalase	Bio polishing, and bio-scoring of cellulosic fabric, bleach clean up	*Bacillus subtilis, Aspergillus niger*
Laundry Industry	Alkaline: Protease, Amylase, Lipase, Cellulase	Stain removal, removal of oil and fats, increase	*Bacillus species, Aspergillus niger*

		in detergents effectiveness	
Pulp and Paper Industry	Xylanase, Cellulase	Enhanced removal of lignin to enhance the bleachability of kraft pulp	*Bacillus species, Aspergillus niger, Trichoderma reesei*
Leather Industry	Protease and Lipase	hydrolyses protein matter into soluble peptides and insoluble fat and oil matter into soluble fatty acids and glycerol	*Bacillus species, Aspergillus*
Starch processing Industry	a-Amylase, Glucoamylase, Xylanase, Glucose Isomerase	Conversion of starch to glucose, production of high fructose syrup	*Bacillus subtilis, Bacillus stearothermophilus, Aspergillus oryzae*
Baking Industry	a-Amylase, Xilanase, Lipase, Protease	Generation of fermentable compounds, reduction in fermentation time	*Bacillus stearothermophilus*
Dairy Industry	Chymosin, Lipases, □-galactosidase, Lactase	Hydrolysis of protein, cheese making, glucose production from lactose	*Bacillus subtilis, Pseudomonas aeruginosa*
Juice Industry	Pectinase, Cellulase, Laccase, a-Amylase	Fruits cell wall breakdown, removal of haze	*Aspergillus Niger, Bacillus Subtilis*
Animal feed Industry	Phytase, Cellulase, Xylanase,	Improvement of nutrient upkeep and availability, improvement of protein digestion	Aspergillus Niger, *Trichoderma reesei*

The value of the enzyme market has been increasing every year. In 2017, the enzyme market reached 7,082 million US dollars and the forecast for the next years looks promising, pointing to 10,519 million US dollars in 2024, representing a compound annual growth rate (CAGR) of 5,7%[65].

The main reason for this increase is that enzymes have been replacing conventional chemicals in various industries, due to environmental norms and regulations regarding the use of chemicals. Carbohydrases represented the largest market share in 2017 and are the most used in the pharmaceutical and food industry. Polymerases and nucleases possess the highest growth potential (HGP). Regarding enzyme sources, it can be from microorganisms, plants, and animals. Microorganisms are the major source of enzymes, due to their easy availability and low production costs, although animal sources possess the HGP. By reaction type, the market can be subdivided into hydrolases, oxidoreductases, transferases, lyases, and others. In 2016, hydrolases held the largest share of the market followed by

oxidoreductases. Transferases possess the HGP, expecting to grow a CAGR of 7.4% up to 2024. In terms of application, the market is segmented into food and beverage, household care, bioenergy, pharmaceutical and biotechnology, feed, and others. The market is dominated by household care followed by food and beverages, but, pharmaceutical and biotechnology-based enzymes possess the HGP which is a good motivation for this work [65].

Although enzymes catalyze an array of chemical reactions in their natural environment, they are not fit for many industrially demanding substrates and conditions. Marine living organisms for example that survive in the most challenging environments like marine extremophiles have the ability to produce biotechnologically useful enzymes, called extremozymes[47]. A powerful biocatalyst like extremozymes can be defined as a biocatalyst with the capacity to be active and stable under severe reaction conditions where there is high substrate/product concentrations, high temperature, and still be able to provide high chemo-, regio- and enantio-selectivity[30][66].Enzymes that naturally don't have these properties need to go through a combination of engineering approaches, to have the same characteristics. One of those approaches is directed evolution, which is an iterative manmade procedure that is achieved when a satisfactory performance level in terms of binding activity, enzymatic activity, or specificity is achieved [67]. Likewise, enzyme immobilization techniques should be considered as a technology that provides, as output, a functional and stable biocatalyst viable for industry and academic interests [68]. The enzymes used in this work and in other works related to the INMARE project were obtained through a targeted sampling campaign in both known and previously unexplored marine biodiversity hotspots, where INMARE amassed a curated catalogue of samples genomic and metagenomic libraries that will be available for future biodiscovery.

As a measure to access the environmental impact of manufacturing processes, Roger Sheldon introduced the Environmental Impact Factor, known as "*E* factor" (kg waste/kg product) in the late 1980s. Later, in 1992, he revealed that the pharmaceutical industry presented the highest *E* factor amongst the chemical industries, meaning that it had a more negative environmental impact than the other industries. That reason demanded an urge to develop "Green chemistry" that is characterized to promote an eco-friendly chemical manufacturing process [69]. Biocatalysts emerged as a great solution for many pharmaceutical industries' environmental problems, with some advantages over chemical synthesis in the production of pharmaceutical intermediates (Table 6) [70].

Table 6 - Environmental impact of chemical industries (adapted from [37]) – The higher E factors in some types of chemical industries, such as the pharmaceutical industry, gives relevance to the Biocatalysis application instead of the standard chemical synthesis to produce both pharmaceuticals and pharmaceutical intermediates.

Industry	**Annual product (tons/year)**	**Annual total waste**	***E* factor (kg waste /kg product)**
Petrochemical Industry	10^6-10^8	10^7	< 0.1
Bulk chemical Industry	10^4-10^6	5 x 10^6	< 1 - 5
Fine chemical industry	10^2-10^4	5 x 10^5	5- >50
Pharmaceutical industry	10-10^3	10^5	> 100

1.3.1. Advantages of using enzymes in API/Fine chemicals manufacturing. Chiral compounds over racemic mixtures

Since enzymes from microbial sources provide an eco-friendlier alternative to chemical synthesis, they are better choice for the production of API's and other pharmaceutical products. Isolated enzymes are often referred to as a step of the biocatalytic process due to their simple implementation, but for more complex reactions that involve more than one enzyme or cofactor, whole-cell biocatalysts are normally preferred, although a cascade of isolated enzymes can be made in sequential bioreactors [30][71]. Both isolated enzymes or whole-cell systems are an attractive alternative for the production of chiral compounds, such as chiral alcohols, which are important starting materials for the synthesis of pharmaceuticals due to their economic advantage, high production yield, and low environmental impact [72][73].

High performance liquid chromatography (HPLC) represents the most popular and highly applicable technology in the field of chiral analysis for a variety of racemic mixtures. HPLC high speed, sensitivity and reproducibility make it the first choice in the analytical laboratories around the world, including those involve drug development in pharmaceutical industries[74]. In this work HPLC could not be used for two reasons: laboratory limitations, and due to the use of a polar solvent, hexane. Instead of HPLC, the chiral analysis was performed by gas chromatography (GC).

An interesting highlight of chiral biomolecules is that in nature, they normally exist in only one of the two enantiomeric forms. Oftentimes, only a single enantiomer of a chiral molecule is desired, and not a racemic mixture, like when the target molecule is a chiral drug that will be used in living systems whereas the use of both enantiomers in a racemic formulation of a chiral drug may lead to undesired side effects or adverse reactions [75]. Regarding the manufacturing of API's, it's worth mentioning the justification for using chiral compounds instead of racemic mixtures, and the best example for that lies in Germany approximately 70 years ago, where a drug called Thalidomide or contergan, was prescribed for insomnias, coughs, colds and headaches. Thalidomide is a chiral compound prescribed as the equimolar racemate consisting of (+)-(R)- and (-)-(S)-thalidomide [76]. The problem urged when thousands of pregnant women took the drug to alleviate symptoms, without knowing that the drug could pass across the placental barrier and harm the fetus. The result was teratogenic deformities in the born children and in some cases, death. (R)-thalidomide was reported to be responsible for sedative effects, whereas (S)-thalidomide and its derivates were reported to be teratogenic. A study [77] suggested that the tragedy of thalidomide could actually been avoided if the (R)-enantiomer had been used, and not both enantiomers[78].

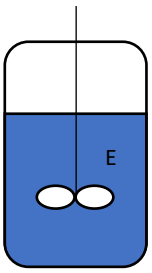
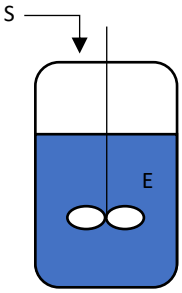
1.4. Bioreactors

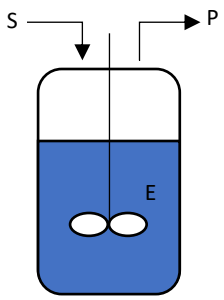
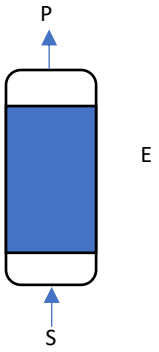
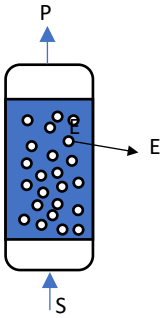
Bioreactors are considered vessels that can provide the best operating conditions for biological systems. The biological systems involved comprise enzymes, microorganisms, animal and cell plants, and tissues. There are numerous types and configurations of bioreactors that were designed according to the demands of several bioprocesses, where there was performed a rigorous control of dissolved oxygen (DO) concentration, pH, temperature, mixing, and nutrients supplementation. In enzymatic processes for example, there is the possibility of having harmful high substrate concentrations, product inhibition and byproducts formations that can affect the overall yield of the process. What can also affect the process yield are physical factors such as shear stress, mass transfer, mixing, pH, and temperature. It is similarly important to have in mind the downstream process requirements because the process is a whole, and the objective in bioreactor design is to integrate the most efficient bioreactor into the system [79][80][81].

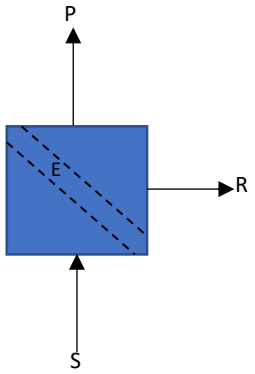
1.4.1 Reactors configuration in biocatalytic bioprocesses

Table 7 sums up the bioreactor configurations that can be applied in bioprocesses such as biocatalysis

Table 7 - Bioreactor configurations applied in Bioprocesses. E – Enzyme/Biocatalyst; P – Product; R – Retentate; S – Substrate [79][80][81][82][83]

Bioreactor configuration	Characteristics/specifications	Illustration
Stirred tank reactor (STR)	• Responsible for 90% of industrial bioprocesses • Consists of a glass, stainless-steel vessel with an impeller driven by an overhead motor. • 75-80% of the volume is liquid • Aspect ratio (H/D) has a wide range	
Fed-batch stirred tank reactor (FB-STR)	• An operation where more nutrients are added continuously or intermittently • Reaction volume increases with time • Commonly used for biocatalytic reactions because substrate concentrations are kept low.	

Continuous stirred tank reactor (CSTR)	• Material is continuously added, and product removed from the reactor (working volume remains constant • Contents and effluent are homogeneous • Biocatalyst must be fed continuously to the reactor, or retained by immobilization and/or partially permeable membranes	
Continuous Packed bed Reactor (CPBR)	• Immobilized enzymes are packed in a column through which a reactant solution is pumped • Ease of operation and scale-up and high bed volume • Lowers shear stress on immobilized enzymes • Long-term enzyme stability	
Continuous fluidized bed Reactor (CFBR)	• The substrate is fed from the lower end of the bed • Mass transfer is better than in packed bed reactors • For an ideal interaction between the biocatalyst and the substrate, the fluidization velocity needs to be optimized	

Membrane Reactor	• The membrane acts as a reactor as well as a separator in the system. • Product is obtained continuously • When the biocatalyst is immobilized on the surface, flushing the substrate solution along the enzymatic gel causes the conversion of substrate into product. • In "Enantioselective membrane reactors", the enzymes in the membrane-associated reaction system can differentiate between chiral molecules.	

1.4.2 Bioreactors scale-up

Bioreactors scale-up process considerations

As it was previously mentioned, scale-up processes face the problem of losing process performance, which is why it is important to consider certain configurations. To attain a certain level of success in biocatalytic processes scale-up is important to understand the interactions between the biocatalyst and the environment inside the reactor. Another important factor to have in mind is the reactor selection since the selected reactor must guarantee that its internal conditions are controllable at all scales [84].

There are several bioreactor types available for enzymatic reactions, each of them with their own advantaged and disadvantages. The considered aspects when choosing the bioreactor type are cost, space, mass-transfer, kinetics, heating and cooling, ease of operation and reusability of the catalyst. The most commonly reactor set-up is the batch reactor, due to equipment flexibility and ease of operation, but at large scale there is a drawback related to low volumetric productivity and that the immobilized enzyme(s) or cells will be exposed to mechanical stress originated from the stirring, which reduces catalytic activity. Other impairment is the decline in enzyme activity as the re-uses increase, which means that either the reaction conditions should be adjusted or more enzymes need to be added to the reactor to maintain the productivity constant [6][84].

Packed-bed reactor (PBR) is another alternative to run enzymatic conversions using immobilized biocatalysts. There are some benefits over stirred tanks like lower investment cost and higher volumetric productivity, and they can be operated in a continuous mode. Other advantages associated with PBR is the reduction of sheer forces, and the shortest residence time can lead to less side reactions. As drawbacks, using a PBR could lead to mass-transfer limitations, difficulty in adjusting pH, performing *situ* product removal and adding reactants. One way to counter these drawbacks is to run several PBRs sequentially with intermittent addition of substrate or removal of product, as it was

proposed by Nielen et.al. in 2008 [85]. Bubble column or air-lift reactors are used to facilitate the contact and/or reaction of a liquid and gaseous phase, or when viscuous reactants are used[6].

As it was previously mentioned, successful scale-up of a biocatalytic process requires a profound understating of the interactions between the biocatalyst and the chemical and physical environment on the reactor. When it comes to choosing a reaction and its operation mode, the goal is to control the environment al all scales, so that accurate predictions can be made from the lab-scale reactor to the industrial scale reactor. When scaling up a reactor, no matter the type or operation mode, it will have their own drawbacks. In stirred tanks for example during a scale-up process, the mixing time is increased. Temperature control of biocatalytic reactions, like the heat transfer from jacketed reactor heating systems is reduced due to lower surface area/unit. The reactants and products need to be controlled in a tight window of pH. At a laboratory scale, one can use buffers, but this is not possible at larger reactors, where acid or alkali will be titrated into the reactor in case pH changes occur. The risk of contamination is a huge economical drawback, because if the batch is contaminated, a lot of material is lost[6].

During scale-up, process modelling, and simulations can allow an efficient evaluation of various process options, since testing out experiments is expensive and time consuming. Since many compounds involved in biocatalytic processes are relatively new, data is not available. The suggested framework is to use software models to estimate the needed parameters, and then to use chemical process design software to simulate the alternative process configurations. Sensitivity and uncertainty analysis are important tools to evaluate the robustness of the process models, and to quantify expected variations in the process outcome and their source. Other important engineering tools for a scale-up process include the regime analysis and windows of operation analysis to determine process constraints, limiting factors and feasible conditions and life cycle assessment that evaluates the environmental impact of the product and the process[6].

There is no ideal reactor for biocatalytic processes since each reactor type has its advantages and disadvantages. What has been studied so far is that some reactors work better with whole cells, while others provide better tools for the activity of isolated enzymes and immobilized enzymes. There are some other top considerations to have in mind when selecting the bioreactor, such as the following parameters (equations 2 to 5)

$$Reaction\ yield = \frac{mass\ of\ product}{mass\ of\ substrate + raw\ materials}\ g/g \qquad 4$$

$$Space - time\ yield = \frac{mass\ product\ \times reaction\ time}{reactor\ working\ volume}\ g.h/L \qquad 5$$

$$Biocatalyst\ yield\ (Y_{PE})\ = \frac{mass\ poduct}{mass\ of\ biocatalyst}\ g/g \qquad 6$$

$$Product\ concentration = \frac{mass\ of\ product}{reactor\ volume}\ g/L \qquad 7$$

For example, Isolated enzymes work well in well-mixed reactors (CSTR and FB-STR), as long as the system provides a biocatalyst recycle mechanism that puts up to good use the advantages that immobilized enzymes have,

such as the increased lifetime cycle usability [86]. One should consider the advantages and disadvantages of using isolated enzymes and whole cells with the active catalyst inside. Table 8 provides them:

Table 8 - Advantages and disadvantages of using isolated and whole cells with the active catalyst inside (adapted [87]

<table>
<tr><th colspan="2">Isolated enzymes</th><th colspan="2">Dead whole cells with active catalyst inside</th></tr>
<tr><th>Advantages</th><th>Disadvantages</th><th>Advantages</th><th>Disadvantages</th></tr>
<tr><td>Better control of the reaction (e.g. pH). No side reactions</td><td>Some enzyme classes require a co-factor regeneration system, like KREDs (Ketoreductases)</td><td>Simple use and reduced costs</td><td>Unwanted side reactions</td></tr>
<tr><td>There is no limitation in substrate and product transport</td><td rowspan="2">Not many enzymes commercially available in pure form or require purification</td><td>Cofactor regeneration employing the cell's metabolism</td><td rowspan="2">Limitation on substrate uptake. The energy required to transport substrate or product across the membrane</td></tr>
<tr><td>Higher substrate and product concentration tolerance</td><td>No need for enzyme isolation and purification</td></tr>
</table>

Whole cells and isolated enzymes

Regarding the scaling up of biocatalytic processes, there are some challenges due to mismatch between the bench-scale and large-scale requirements. One of those challenges is to have the same product concentration when moving from the laboratory to full-scale implementation, because when working at large-scale, enzymes are operating outside their environment, resulting in activity and stability problems. There has been stated [88] that miniatures scales should be used to help target where the protein engineering efforts should be placed. The bioreactors used in this work, can be considered miniature scales due to their small volumes, and can help developing new models for scale-up.

Considering one step-reactions, isolated enzymes are theoretically better when compared to whole-cells, because no side-reactions should occur, and substrates do not need to be transported across cell membranes. On multi-step bioconversion processes, whole-cells have the advantage. Literature implies that pure enzymes present some several advantages such as higher specificity, better tolerance to co-solvents and simpler apparatus and procedures, although the process of enzyme isolation being quite expensive and time consuming. The major advantage of using whole-cell biocatalysts is that the cell provides a natural environment for the enzyme that allows for efficient co-factor regeneration, counteracts conformational changes in the protein structures that would result in loss of activity towards non-conventional medium [89].

Regarding the improvement of biocatalytic activity in isolated enzymes there should be a compromise between enzymatic stability and activity because modification in the protein structure could make the access of the substrate to the active site of the enzyme difficult impairing enzyme activity. To improve enzymes stability, enantioselectivity and activity, there should be applied tailor made immobilization techniques. Regarding enzyme engineering, these can be achieved by two complementary strategies, rational redesign and directed evolution. Rational redesign enhances the understanding of enzyme binding and catalytic mechanisms and directed evolution detects mutants that possess

improved properties. A combination of both strategies can be the most productive route to improve the properties and function of an enzyme [90].

Whole cell catalysts are a protected environment and most of the times more stable than isolated enzymes. Substrate transference is one of the limitations inherent to whole cells, and a way to overcome substrate transference requires permeabilization of the cell wall and membranes by chemicals (e.g, adding detergents or solvents) or physical (e.g temperature shock) treatment, even though these treatments can damage the cells. Many recent studies, regarding biocatalysis with whole cells used an organic-aqueous system, where the contact with an organic solvent influences the physiology of the bacterial cells, which means that solvent toxicity is probably the major factor influencing cell behavior in an organic-aqueous system, besides substrate concentration, cell adaptation ability and membrane composition [91].

Whole cells are becoming more common in the production of compounds with industrial interest, as an alternative to traditional chemical synthesis. Aqueous-organic two-liquid phase system, as the studied system in this work, enable higher overall bioconversions, and allow overcoming substrate and product inhibition, some of those systems even reach industrial scale implementation. The scale-up of these processes requires constancy on parameters such as volumetric power input, impeller tip speed, Reynolds number, mixing time, and many others. Like in other previous works, the chosen parameter to be evaluated in this study when the reactor volume is increased was the droplet size in the interfacial area of the studied biphasic system, for a constant agitation across the reactor volumes [92].

Continuous agitated reactors

Characterization of a continuous agitated cell reactor for oxygen dependent Biocatalysis

Typically, biocatalytic oxidations have been dominated by batch processes, mainly due to their great flexibility for manufacturing. But recently, there has been a drive for continuous processing due to cost reduction, improvement in process control and waste reduction was the motivation for some authors to develop a novel continuous agitated cell reactor [93] (ACR). They used the oxidation of glucose to glucono-1,5-lactone by glucose oxidase as a model reaction to study the ACR. The results showed that the 100mL ACR was able to increase the overall reaction rate (more than double), solely due to an increased OTR, (comparing to a batch reactor of similar volume), which marks a reduction in processing times and more efficient use of the biocatalyst.

Scaling up Biocatalysis in continuous processes

The continuous operation has been gaining track over batch and fed-batch processes for the pharmaceutical and fine chemical industry, mainly due to footprint reduction and more sustainable manufacture. Continuous flow reactors look especially attractive for biocatalytic processes, due to enhanced mass transfer, enhanced economy for large-scale production in smaller equipment with smaller reaction times, improvements in space-time yield in comparison to bath processes [95] [96].

The following study [96] aimed to evaluate if the scale-up of a biocatalytic oxidase process can benefit from the improved mixing and mass transfer that is characteristic of continuous flow bioreactors, in comparison to batch bioreactors.

The results in batch bioreactors showed that the smallest of the scale (250mL) provided a higher reaction rate compared with the bigger scales, 1L, and 4L. This is evidence of the practical problems of scaling up gas/liquid reactions in batch reactors. As the size of the reactor increases, the complications of having a constant gas/liquid dispersion and

efficient mixing energy distribution also increases. The problem can be managed by increasing the number of small-batch reactors, but then another problem arises, the capital and operating costs. The authors presented a plug flow bioreactor model called “Coflore®”. It uses transverse mixing lateral movements of the agitators, which brings the advantage of being self-baffling and eliminates the problems of phase separation due to centrifugal action.

When comparing the reaction rates of 1L Coflore® reactor with the 1L batch rector, at the same conditions (enzyme load, agitation speed, and oxygen flow), the results show that the Coflore® tubular reactor is three times faster than a 1L batch reactor.

When the Coflore reactor is scaled up to 10L, the reaction rate remains practically unaffected, but the oxygen consumption is reduced by 70%, this suggests that in the batch reactor, the oxygen is being used in large excess and wasted since it remains the same during scale-up.

One can assume, based on the presented results, that the scale-up of batch reactors does not perform as well as flow reactors for oxidase reactions. Such an assumption can be explained by the fact that the batch alters vessel diameter, height, and agitator speed (relative to the process fluid during scale-up). The Coflore® when scaled-up from 1L scale to a 10L scale, does not seem to affect the enantioselectivity of the oxidase involved as well as the enantiomeric excess and also shows that it can be quite effective on oxygen consumption management since it can reduce oxygen consumption at larger scales, as observed on the following table 9:

Table 9 - Comparison between the scale-up of an oxidase reaction in batch and continuous flow conditions (adapted from [96])

Performance Parameter	**1-L batch reactor**	**4-L batch reactor**	**1-L ATR**	**10-L ATR**
Velocity (m/s)	N/A	N/A	0.00002	0.0002
Pressure drop (mm water gauge)	N/A	N/A	<1[a]	<1[a]
Reaction rate at 50% conversion (%/h)	4	2.4	9.8	10.3
Conversion at 4h (%)	20.8	20.1	55.7	54.6
Conversion at 8h (%)	35.9	30.4	94.1	89.7
Oxygen consumption ((L/h)/L)	0.25	0.25	0.25	0.075
[a] by calculation				

Scale-up in most of the continuous processes is led by increasing the total reactor volume and the flow rate to maintain the same residence time, that is established during development. An alternative for continuous processing scale-up is to use several microreactors, that can operate be operated in parallel [97]. LaPorte et al. [98] described the development of a scaled-up continuous process in small reactors to prepare 6-hydroxybuspirone, an active pharmaceutical ingredient. Small reactors have the advantages of having a small hold-up volume and great heat transfer

characteristics, which results in a better reaction control, and the continuous processes provide better safety quality and economics when compared to batch processes. The authors created a replica of a trickle bed column where the tubes were jacketed to affect the temperature control. The examined operation parameters were: Flow rates for the enolate solution; Oxygen Flow rates; Oxygen pressure; Heat transfer rates; Operation temperature within the oxidation temperature.

The results of the study had 2 main achievements. The first was a successful change from a batch reaction system to a fully continuous process to produce 6-hydroxybuspirone, which provided a faster reaction and a better mass transfer. The second was the successful scale-up to a pilot plant scale of the continuous process by "numbering-up", which is defined as multiple operations in parallel [99].

Successful scale-up of a monooxygenase enzyme to form a chiral Sulfoxide

Oxygenases are often used to synthesize compounds that are not available through chemical routes. There are numerous small-scale industrial applications, for example, mammalian hydroxylases and their bacterial and fungal equivalents are used to synthesize a wide range of intermediates of human drug metabolism. A two-liquid phase approach was tested to enhance and stabilize biocatalyst performance by overcoming reactant toxicity as well as mass transfer limitations and it showed promising results in the scale up from a glass tube to a stirred-tank photobioreactor setting[100][101].

For the preparation of high purity chiral sulfoxides, biocatalysis methods can take two paths. Either by employ whole-cell systems or isolated enzymes. Modern biotechnology has been showing a preference for the use of isolated enzymes since they can be genetically engineered with directed evolution to increase stability and selectivity [67]. DoE was applied to investigate substrate concentration, enzyme concentration, buffer charge, enzyme type, buffer type. On a successful scale-up of a monooxygenase to form a chiral sulfoxide, when scaling-up to 100mL jacketed vessels with overhead stirring, two problems urged:

- Variable reaction times between days – resulting from slow airflow into the vessel, causing slow oxygen uptake
- Loss of solvent over time

To surpass these obstacles, and improve conversion, the second batch campaign was sparged with a stream of air (using a standard propylene tube to bubble compressed air into the reaction vessel), while simultaneously monitoring the level of buffer in the reaction system. These conditions resulted in 98% of conversion in 21 hours. The last campaign of the study aimed to improve mass transfer while reducing enzyme loading at the same time. With air piped into the vessel and with highly effective agitation, the reaction was complete in just 6 hours. The enzyme load was reduced by 5% wt/wt, with a negligible impact on the product quality or diastereomeric ratio. All batches were on a 1kg scale in 100L reactor vessels [102].

Another successful biocatalytic scale-up was the asymmetric synthesis of an antiepileptic drug. The API (*R)*-2-propylsuccinic acid 4-tert-butyl ester was produced at 1 kg scale in a 10L vessel with a yield of 42% and 99% ee. The resolution without any co-solvent was prioritized to simplify the work-up. The enzymes conversions rate was evaluated either with and without a few co-solvents and also in a range of pH, to evaluate the optimal conditions that provided the best conversion rates, ee, and also E value.

A key factor to ensure the successful enzymatic scale-up from screening conditions to agitated vessels was the change from the phosphate buffer to aqueous media with pH control (limiting the overall volume of reaction for

industrial perspectives). When performing the reaction at pH 8.5, the 42% yield and 97% ee was obtained 3 hours faster (17 hours vs 20 hours) when compared to pH 8.0. This gives information about the best possible pH range to optimize the process.

The enzymatic resolution was then scaled up from 250ml to a 10L vessel, presenting the same yield and ee of the laboratory scale, respectively 42% and 97%, showing consistent results across both scales. There is room for improvement in the process, especially in the final step where the aim would be focused on minimizing the suspected substrate degradation and improvement of the isolation of the API, to apply this bio-catalytic route as a commercially viable route for the production of Brivaracetam® [103].

Moccia et al. [104] reported the development of a new process to manufacture (S)-Pregabalin which is currently manufactured by Pfizer and other generic pharmaceutical producers. The method designed by the authors has a 65% yield which is a great achievement when comparing to the previous methods that only range between 25-30% yield. Having also in mind that all reagents can be obtained at a relatively low price, and the solvents and catalysts are recycled, the proposed process demonstrates that it is cost efficient and competitive for the industrial scale production of (S)-Pregabalin.

Scale-up of lipase-catalyzed polyester synthesis

In a study [48], some authors developed a successful scale-up for the enzymatic reduction of glycerol adipate on a 500g scale in a heated, solvent-free system, where several reaction conditions that influence the substrate conversion rate were assessed, such as temperature, pressure, enzyme concentration, reactants ratio, stirrer type, stirring rate and reaction time. The results showed that the optimal temperature was around 60ºC and enzyme concentration between 1 and 3 wt %. As it was previously mentioned, mixing influences the mass transport in the reaction mixture. Mass transport is important because the substrate must reach the active site of the enzyme. Conversion rates were not affected by the tested stirrer types (pitched blade, anchor, and helicon ribbon stirrers). On the other hand, mixing is strongly influenced by the stirrer type. The results of the work showed a protein content of 0.48g/kg final product, implying that 45% of the enzyme carrier was destroyed after the reaction, and therefore, in future process development, the increase in mechanical stability should be considered.

Future perspectives for scale-up

- Greater emphasis on stability studies in an industrial environment.
- Development of a suitable methodology to translate laboratory reactions into the industrial process, bioprocess engineers are still building the necessary reaction framework to evaluate all the options of biocatalysts format and operational mode
- Reaction engineers will play an important role in the research that leads to industrially viable processes for a sustainable future.

2. Materials & Methods

2.3. Enzymes

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 program (Table 10).

Table 10 – Designation and characteristics of the used Biocatalysts

Designation	Characteristics
EH	Lyophilized cells
Abo	Wet cell extract

2.4. Reagents

The substrate methyl-2-phelylpropanoate (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.

2.5. 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. The 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 kinetic parameters, Km and Vmax (Figures A.1 – A.3) were determined by the Michaelis-Menten equation, where V_0 units were (umol/min*g dry cell) and the substrate concentration in (mM/mL).

The next step was to evaluate the scale-up effects on the hydrolysis of the substrate *p*-NPB. For that, each micro-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 11).

Table 11 - Description of the enzyme, substrate and buffer volumes and concentration in 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 dry cells	$8.4x10^{-6}$ g dry cells	$16.8x10^{-6}$ g dry cells
EH mass of cells	$5.0x10^{-7}$ g dry cells	$10.0x10^{-7}$ g dry cells	$20.0x10^{-7}$ g dry 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 aluminum 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.

2.5.1. 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 EH 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 GC. To inactivate the enzymes (suicide assays), there was added hexane.

2.5.2. 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

2.5.3. Reactors mixing efficiency

The mixing efficiency at the 3 reactor volumes was measured by naked-eye following a reaction of crystal violet with NaOH. The objective of this experiment was to determine the reaction time for each reactor where the reaction started when the NaOH was added and finished when the solution was transparent to the naked-eye. 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 $1x10^{-5}M$[105]. The time that the solution took to became transparent was determined by observing RGB channels on 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 efficiency6.

2.5.4. Interfacial area for different reactor volumes

The interfacial area between the aqueous and organic phases was also evaluated for all scales. For that experiment, 3 reactions were prepared for the 3 scales in duplicate, and the samples of the enantioselective hydrolysis of methyl-2-phenylpropanoate (50µL) were taken at approximately 25 % and 50% substrate conversion (Table 12).

Table 12 – Sampling time 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	0.75	3.5(3)
20	2.1(3)	4
40	2.2	5

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 ImageJ, 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.

2.6. Bioreactors volumes & Geometries

The reactors used for the experiments were acquired from Phenomenex. The reactors model's information is provided in Table 13 and the screw caps information in Table 14.

Table 13 - Verex vials dimensions and characteristics provided by the manufacturer - Phenomenex

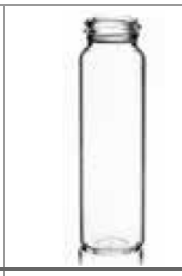
VEREX vials			
Total Volume	10 mL	20 mL	40 mL
Dimension	23 x 46 mm	23 x 75 mm	28 x 95 mm
Neck	18 mm	18 mm	24 mm

Table 14 - 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

The magnetic stirrer bar used for all the reactors was acquired from Kartell, with a length of 12 mm and a diameter of 3mm.

2.7. Enzymatic activity

2.7.1. Enzymatic activity towards *p*-nitrophenyl butyrate

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 5. The reactions resulting solution showed a yellow color [106].

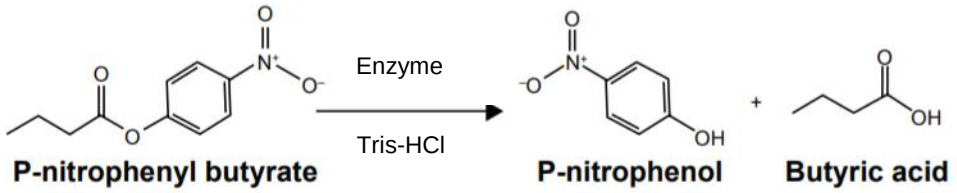


Figure 5 - Hydrolysis of p-nitrophenyl butyrate (*p*-NPB) (adapted from [119])

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).

$$\frac{dAbs}{dt} = \frac{\varepsilon \times 1 \times d[product]}{dt} \qquad 8$$

2.7.2. Enzymatic hydrolysis of methyl-2-phenylpropanoate

The enantioselectivity of the biocatalyst EH 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 reactor scale 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 6).

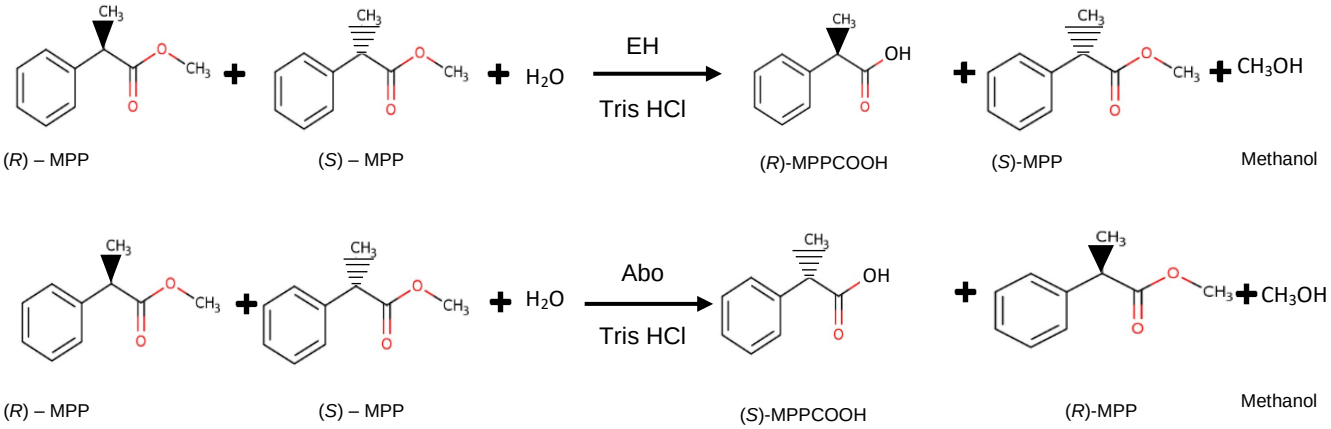


Figure 6 - Hydrolysis of methyl-2-phenylpropanoate to methyl 2-phenylpropanoic acid

2.8. Analytical methods

2.8.1. Samples preparation for the gas chromatography analysis

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 reaction of the biphasic organic system, the reaction volumes were 1, 2, and 4mL for the respective 10, 20, and 40mL scales. 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. After each selected time point (30min,1h, 2h, 3h, 4h, 5h and 6h) 1, 2 and 4mL of hexane was added to the reaction mixture of the 10, 20 and 40 mL reactor respectively. The following step was the transfer from all the volume inside the reactor to eppendorfs and falcons that were submitted to 1minute of vortex and then centrifuged for 10 minutes at 10000 rpm. After the centrifugation, the eppendorfs and falcons settled in a support for 10 minutes. Following that, 200 µL of each sample was added to the inserts inside the vials, and these vials were then analyzed by gas chromatography.

3. Results and Discussion

3.1. Storage of lyophilized and wet paste cells, how many 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 handle. In particular to also assess what was the best storage temperature for these enzymes. This experiment took 84 days for EH enzyme and 91 days for Abo enzyme (Figures 7 and 8 respectively), where enzymes were stored at +4ºC and -16ºC. A cycle of freezing and thawing refers to the act of removing the enzymes from the storage temperature, hydrate them in the case of the lyophilized EH cells with Tris-HCl 20mM, test their activity towards the substrate and freeze them again at both temperatures. It should be noted that the same Abo and EH cells stock was used during the whole experiment, which was divided into 2 eppendorfs and each one stored at the respective temperature (+4ºC and -16ºC). The enzyme activity is expressed as µmol/min.mg of cells. Each point in figures 7 and 8 correspond to a freezing and thawing cycle.

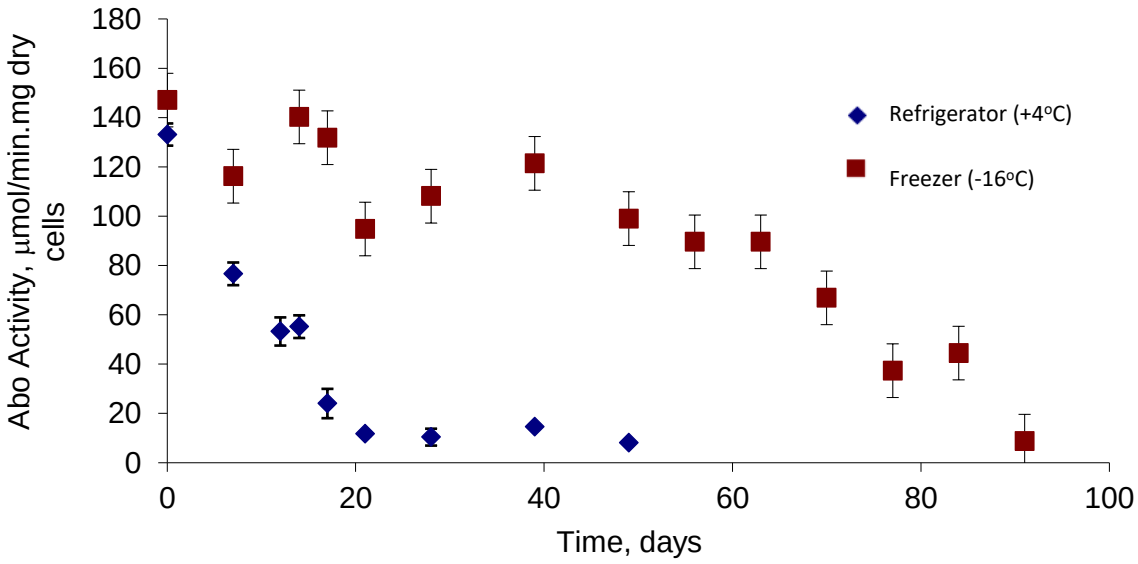


Figure 7 – Abo activity during freezing and thawing cycles

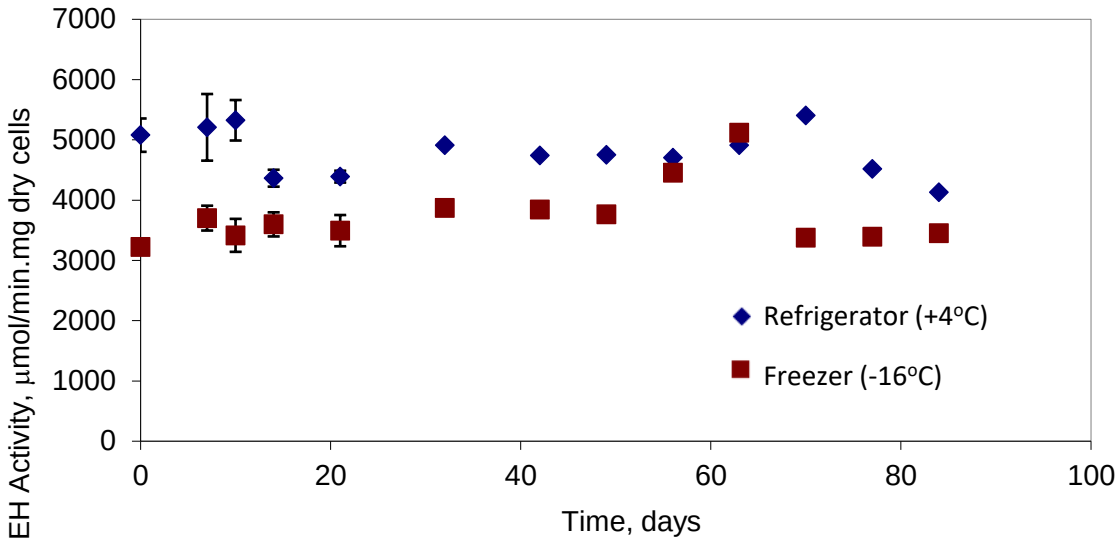


Figure 8 - EH activity during freezing and thawing cycles

According to Figure 7, 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 8 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. Effect of the bioreactor volume on enzyme activity

The activity of EH and Abo, was assessed by measuring the hydrolysis of *p*-NPB, where the reaction velocity curves are available in the appendix section (Figures A. 1 e A. 2). In this study, the aim was to evaluate the changes in the biocatalyst's efficiency at different reactor volumes. In order to try to have the same reaction conditions inside the reactors, temperature and light effects on the enzymes activity were also assessed.

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. When the reactors system was at 30ºC with dark conditions the reactor volume increase effects were more noticeable from

the 10mL to the 20mL scale (loss of 21.06% of activity between reactors, meaning that in the 10 mL reactor, the enzyme was 21.06% more efficient than at the 20 mL reactor) comparing to the following reactors, 20mL and 40mL (loss of 3.91% of the activity between reactors) (Figure 9). At 22ºC, under light conditions, the biggest difference in activity was between the reactors of 20 to 40 mL, which accounts for a loss of 12.82 % (Table 15).

Table 15 - Abo activity at 22ºC and 30ºC under dark conditions

Reactor Volume (mL)	**Activity – 30ºC and dark conditions (A)**	**Activity – 22ºC and dark conditions (B)**
10	272.34 U	203.32 U
20	215.41 U	200.29 U
40	206.99 U	174.62 U

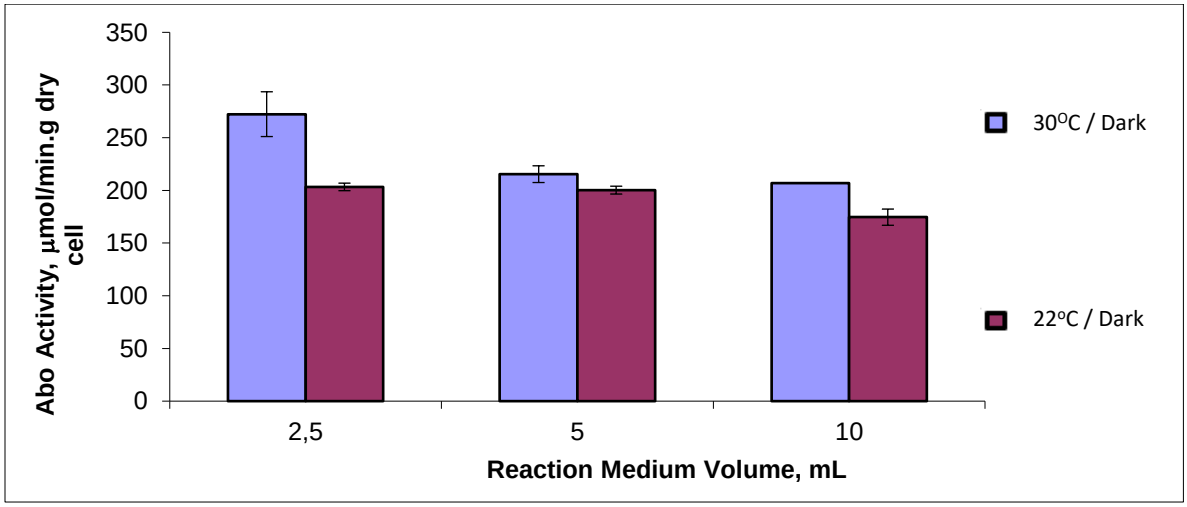


Figure 9 – Abo activity at 22ºC and 30ºC under dark conditions. The activity of the enzyme is expressed as µmol/min*g dry cells

Regarding the impact of reactor volume increase for EH activity, at 30ºC and dark conditions, it was found that the activity of the enzymes decreased when increasing the reactor volume, unlike Abo that maintained its activity in the different bioreactors. Volume increases at 30ºc under dark conditions, caused only a small reduction in the activity of EH (Figure 10 and Table 16). At 22ºC under light conditions, the activity increased 13.34% between 10 and 20 mL and 8.19 % between 20 and 40 mL. The biggest difference in activity is between the system at 30ºC under dark conditions and the system at 22ºC under light, being the biggest activity difference identified between the 10 and 20 mL where it reaches a 40.84% reduction in the activity.

Table 16 – EH activity at 22ºC and 30ºC under dark conditions

Reactor volume (mL)	Activity – 30ºC and dark conditions	Activity – 22ºC and dark conditions
10	12896.01 U	7629.87 U
20	12682.01 U	8648.17 U
40	12362.26 U	9356.55 U

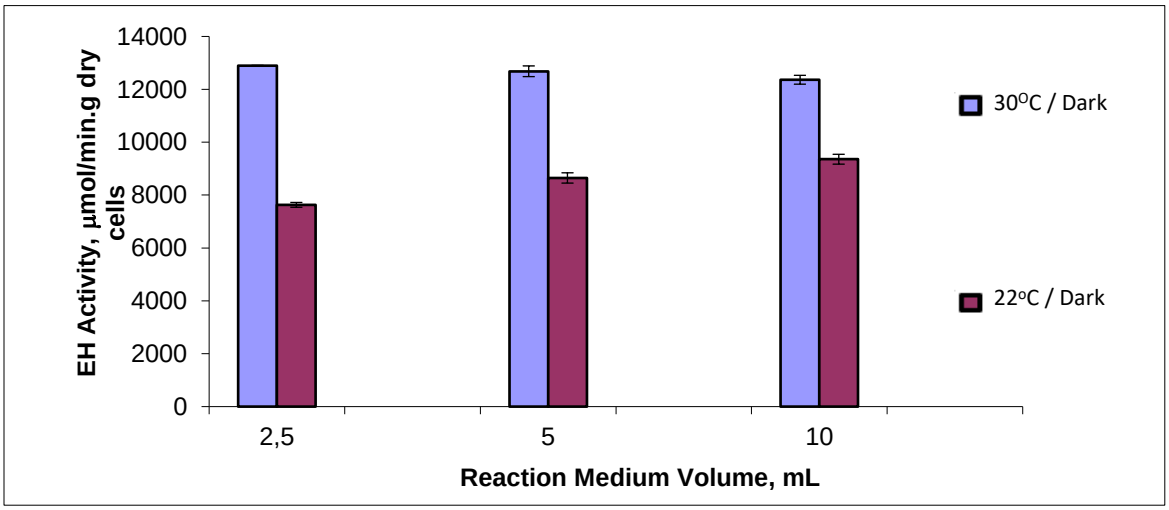


Figure 10 EH activity at 22ºC and 30ºC under dark conditions. The activity of the enzyme is expressed as µmol/min*g dry cells

Both enzymes lost activity when the reactor volume was increased at 30ºC under dark conditions (Figure 11). 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.

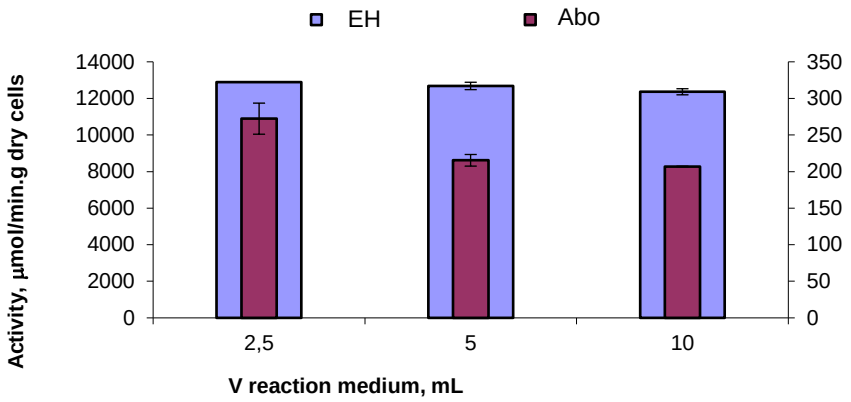


Figure 11 - Activity comparison between both esterases during the enzymatic hydrolysis of p-NPB at 30ºC under dark conditions. The activity of the esterases is expressed as µmol/min*g dry cells

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%) (Figure 12).

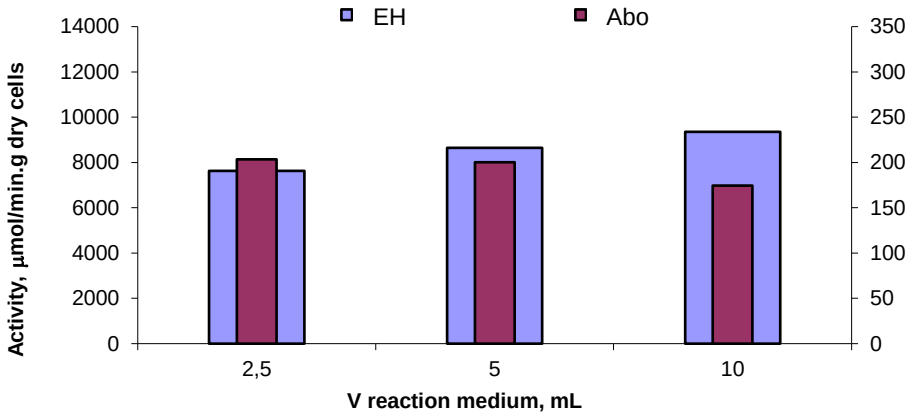


Figure 12 - Activity comparison between both esterases during the enzymatic hydrolysis of p-NPB at 22ºC under light conditions. The activity of the esterases is expressed as µmol/min*g dry cells

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.

3.3. Reactor mixing efficiency

Performance and scale-up in a bioreactor is affected by mixing efficiency. Scale-up on a system's mixing efficiency affects its transfer processes, and one of the most used parameters (parameter under study) to characterize stirring in a bioreactor mixing efficiency is mixing time, which can be defined as the time a liquid needs to be stirred to obtain a certain degree of homogeneity after adding a pulse signal of a tracer [107].

Although mixing time is vastly used in process industries, there has not been found in the literature a standard definition. One of first attempts was performed by Kramers et al. (1953) [108] who measured the time of an injected tracer solution into the batch stirred tank reactor. The authors defined mixing time as the interval from the time the tracer is injected to the time when the tracer concentration at the specific location is within 0.1% of the final mean concentration in the tank. Nere et al. (2003) [109] and Ghotli et al. (2013) [110], determined the mixing time by adding a tracer input at some locations in the reactor and measuring the tracer concentration as a function of time, where the tracer is distributed through the vessel, mixes with the fluid in the reactor until the final uniform concentration is achieved. The authors considered that the mixing time can be defined as the time required to achieve a certain degree of uniformity. The measurement techniques used can be: visual; conductivity; thermal; electrical impedance/resistance tomography; laser-induced fluorescence (LIF); liquid-crystal thermography and computer tomography with coherent light. Crystal Violet can be used as a tracer for mixing efficiency studies. In a polymerization reactor system, the study of the mixing time was estimated by injection of crystal violet as a tracer dye [111].

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. Figure 14 is a representation of 7-time points, where 3 selected pixelized areas were continuously monitored to provide an RGB time-curve. The data for the RGB channels were plotted into a linear regression by fitting a linear equation to the observed data. The linear regression curves for the pixelized areas at each scale are provided in figures 15-17 and the original data from the RGB curves is in the annexes (Figures A. 4 – A. 6). Figure 13 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 [112].

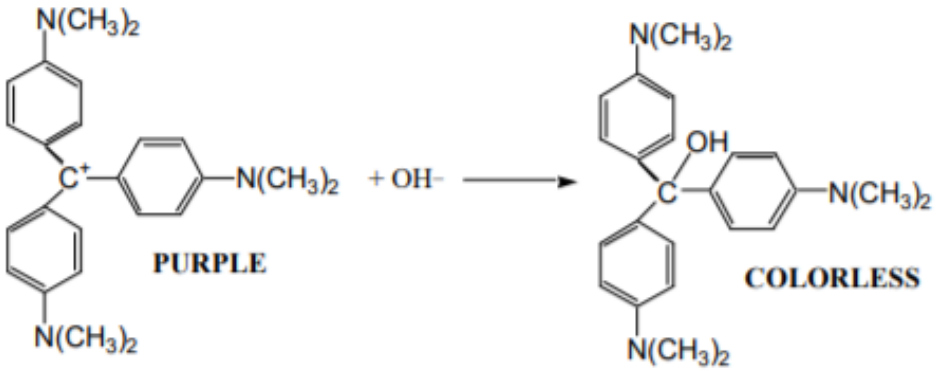


Figure 13 - Reaction between crystal violet and NaOH used to study mixing efficiency of the biocatalytic system (adapted from [112])

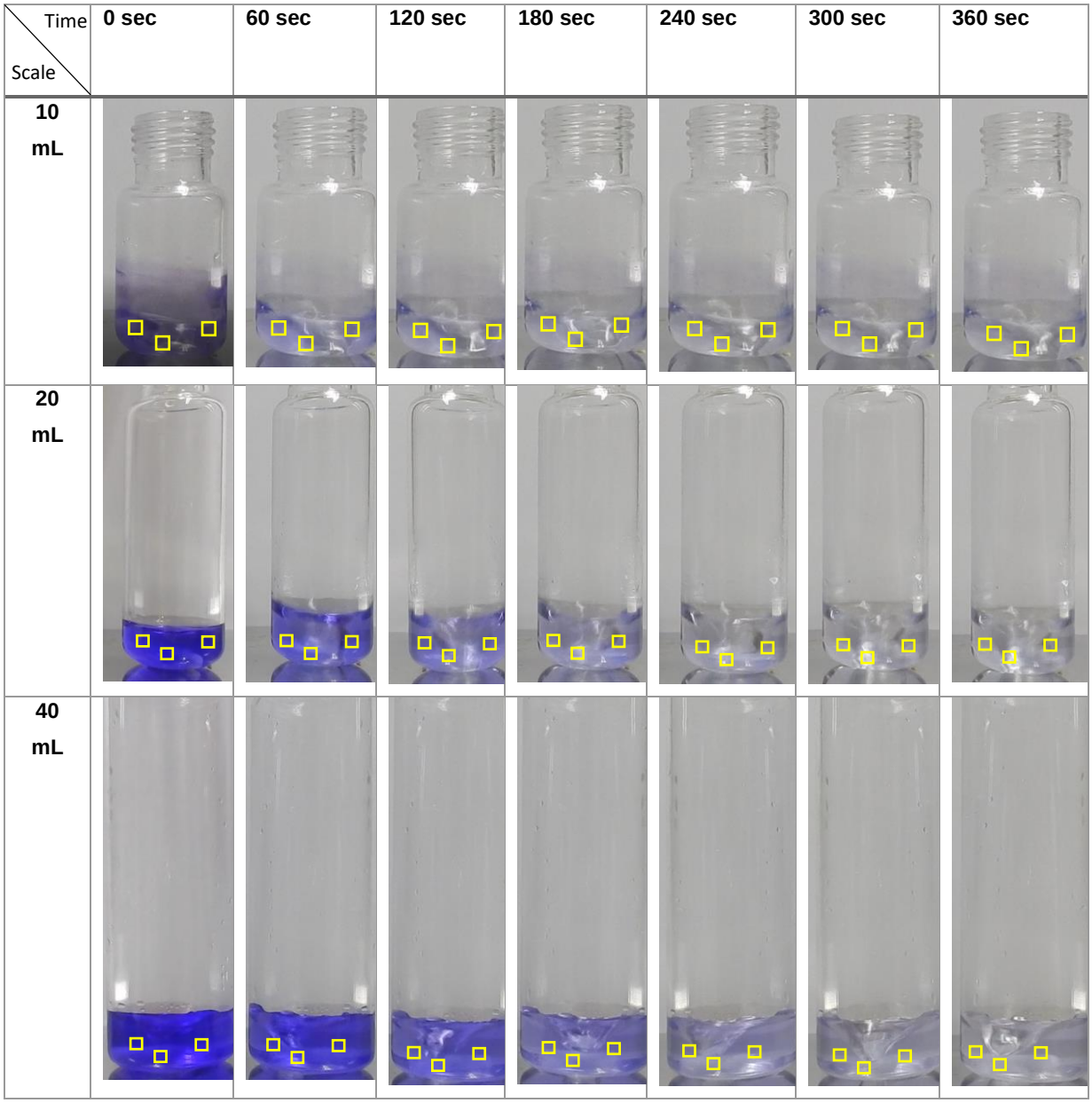


Figure 14 – Colour changes observed during the reaction between crystal violet and NaOH allowed the assessment of mixing efficiency in the bioreactors tested

According to the naked-eye observation, the reaction mixture inside the 10mL reactor was the fastest to become transparent after 280 seconds of reaction, which is 40 seconds faster than the reaction mixture inside the 20 mL reactor (320 seconds) and 80 seconds faster than the reaction mixture inside the 40 mL reactor (>360 seconds). These observations can be confirmed by looking at the following graphs presented in Figures 15,16 and 17. These figures represents the linear regression of the output data from the image processing macro that is provided in the annexes, where each pixelized area is evaluated for each color channel, meaning that the image software analyses Red, Green, and Blue at each selected time point for 360 seconds, where the main goal was to identify where the channels reach 0.

According to the results, the smallest scale again appeared to be the fastest where the RGB channels reach 0, which probably indicates a faster reactor mixing time. The data provided by the linear regression is summarized in Figure 18 and Table 17. Table 18 represents the comparison of the reaction time by 2 different methods, naked-eye and inflexion point.

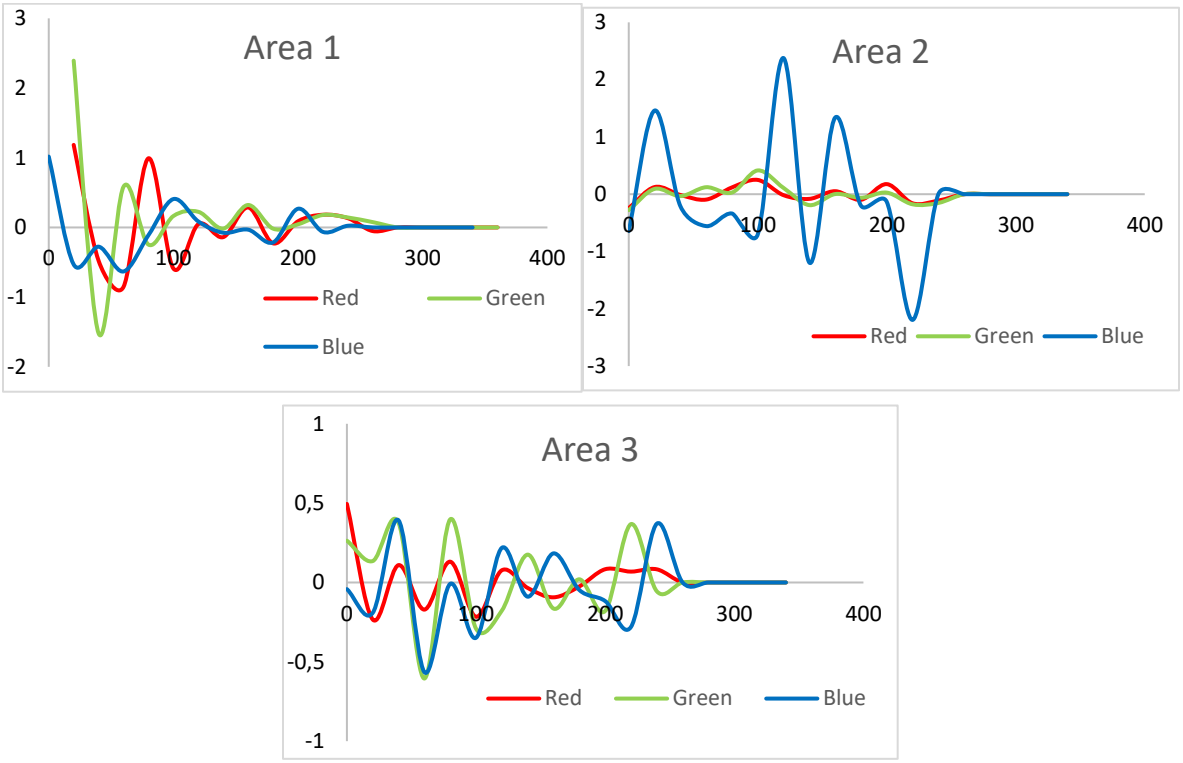


Figure 15 - RGB channels data at the 3 pixelized areas for 10 mL scale

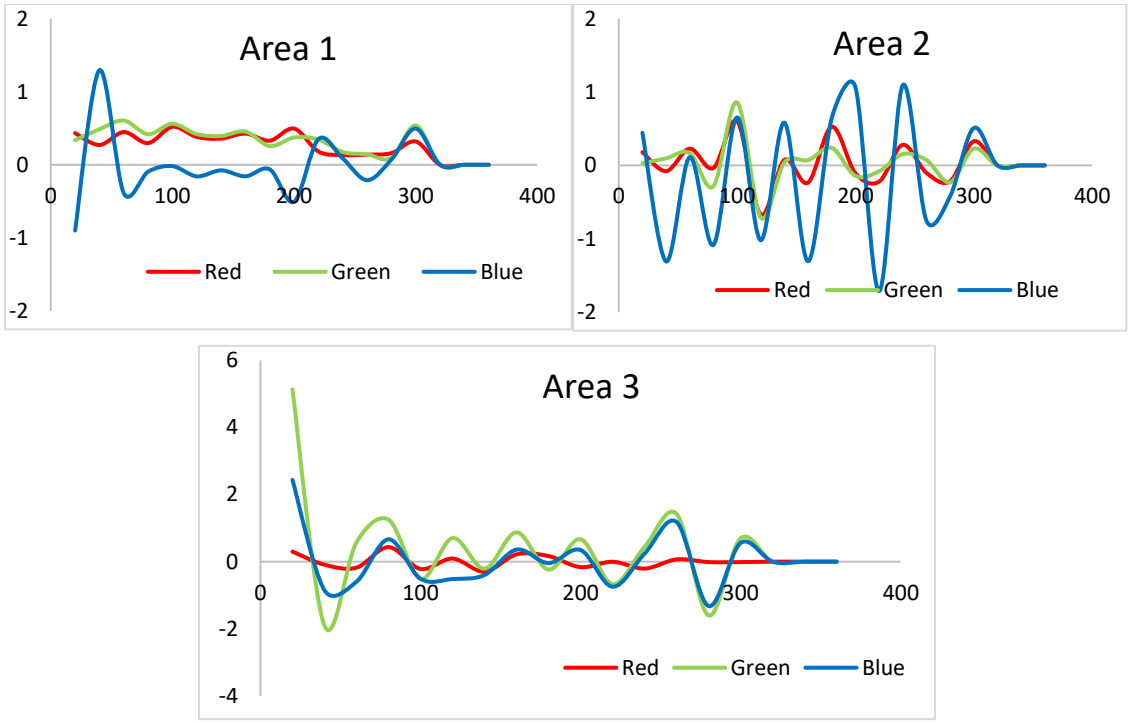


Figure 16 - RGB channels data at the 3 pixelized areas for 20 mL scale

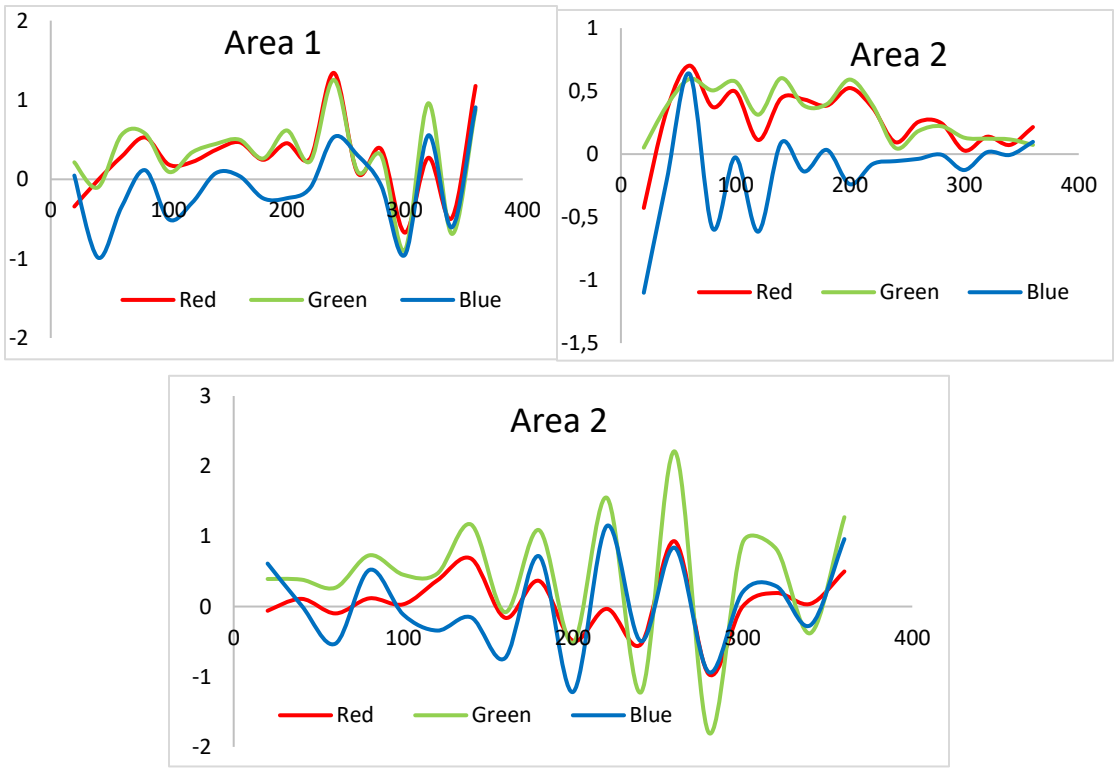


Figure 17 - RGB channels data at the 3 pixelized areas for 40 mL scale

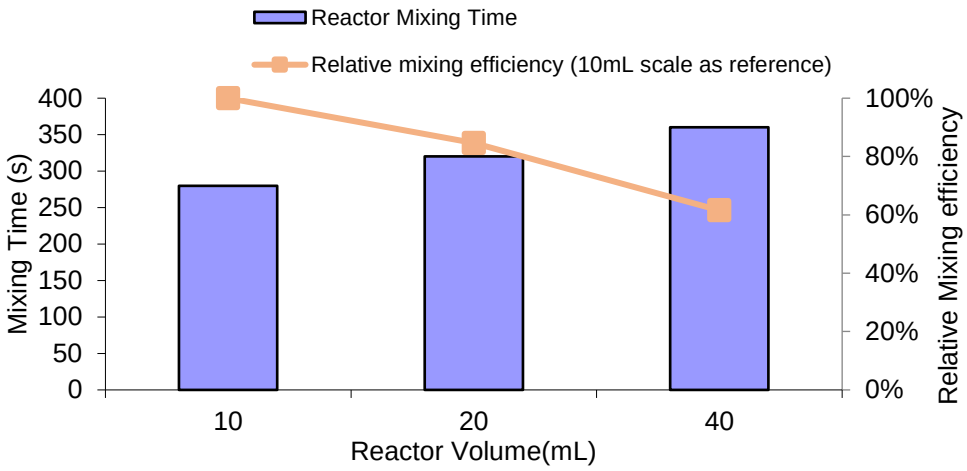


Figure 18 - Reactors mixing time and relative mixing efficiency, where the 10mL scale is the reference

Table 17 - Reactors mixing time and relative mixing efficiency, where the 10mL 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 18 – Mixing time determined by the 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

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. 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 (this can be consulted in the annexes from Figure A. 6). Table 18 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. Figure 18 is a model that was presented by a few authors that aims to identify the most important optically observed parameters in cylindrical containers

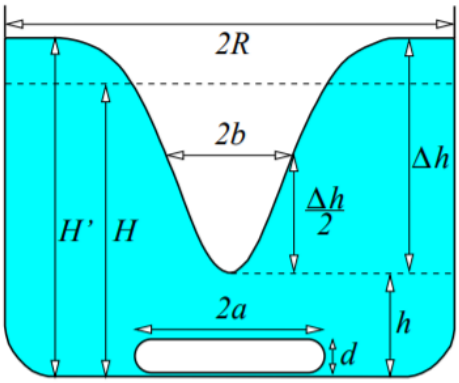


Figure 19 – Important optically observable parameters for the reactor mixing efficiency problem. R, is the radius of the reactor, a and d, are the radius and the height of the stirrer respectively and H is the height of the liquid. When stirring, the observed parameters are the height of the liquid is displaced H', the distance h between the funnel's deepest point and the bottom of the container, the funnel's depth and halfwidth $\Delta h = H' - h$ and b, respectively (adapted from [113])

The presented model in figure 19, and the values in Table 19 are based in figure 20. The reason why it could not be used the same model is because the visualized vortex ended at the bottom of the reactor in the studied system, while in the presented model the bottom of the vortex is not as practically half-way to the bottom of the reactor. This can possibly be explained by the proximity of the reaction volume at steady state (H) to the reactor to the reactor bottom, and the high magnetic agitator rpm's that increase the vortex.

Table 19 – Important optically observed parameters for the reaction system under study. This table is related to Figure 19

Reactors / **Parameters**	10 mL	20 mL	40 mL
2R	23 mm	23 mm	28 mm
H'	5.52 mm	9 mm	11.04 mm
H= **Δ**h	4.6 mm	7.5 mm	9.5 mm
2b	8.05 mm	8.20 mm	14 mm
Δh/2	2.76 mm	4.5 mm	5.52 mm
2a	12 mm		
d	3 mm		

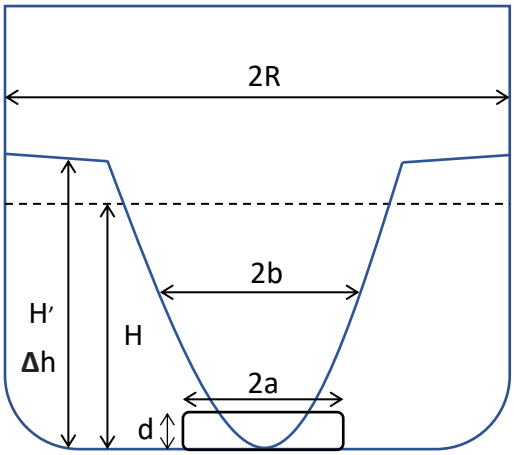


Figure 20 – Important optically observed parameters for the reaction system under study.

As expected, the parameter values in Table 19, vary in accordance with the reactor size and reaction volume, which means that all the parameters values increase except the parameters 2a and d, that are relative to the magnetic agitator. The importance of this parameters should be further investigated to understand if by changing the parameters values there is a significant alteration in the reactors mixing efficiency. The authors that designed the model in figure 18 [113] suggest that there can be used a narrow cylinder of nearly the same diameter as the length of the bar, and after estimate the magnitude of the error and compare the parameters of the vortex in the modified and original setups. So basically, there can be made some alterations to the system (reactor or agitator) and find out if there are improvements in the mixing efficiency of the system.

3.4. 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 [48].

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 an aqueous-organic biphasic system 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 [48].

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 were smaller, corresponding to 25 and 50% of substrate conversion, respectively.

To obtain the number of pixels and also the μm^2 area, 2 measures were needed: The Horizontal Field Width (HFW) and the Vertical Field Width (VFW) (Table 20).

$$Area\ (Pixels^2 or\ \mu m^2) = HFW \times VFW \tag{9}$$

Table 20 – Horizontal Field Width and Vertical Field Width of the processed images in pixels and μm

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

Applying the equation (9) with the data provided in Table 23, the total area obtained was 480000 μm^2. Then the bubble pixelized area is given by equation (10)

$$Bubble\ pixelized\ area = \pi \times r^2 \tag{10}$$

And the maximum and minimum bubble radius by equation (11) and (12) respectively:

$$maximum\ radius = \left(\frac{maximum\ area}{\pi}\right)^{1/2} \tag{11}$$

$$minimum\ radius = \left(\frac{minimum\ area}{\pi}\right)^{1/2} \tag{12}$$

To confirm that the image software was providing a valid output, each image went through the same manual methodology of finding the substrate bubbles with the biggest and smallest radius. The software used was “Fiji”, an image processing package from “ImageJ”

$$Radius\ (Pixels) = \frac{HFW\ (\mu m^2)}{HFW\ (Pixels)} = Radius\ (\mu m^2) \tag{13}$$

So, if the manual search identifies that the biggest substrate bubble is, i.e, 160 pixels, which then gives 50.05 μm applying equation (13), it means that the biggest bubble radius that the image software reads, must not be higher than 50.05 μm. The same applies to the smallest bubble found. Fortunately, the software always confirmed the manual readings, meaning that almost all bubbles were correctly measured.

The following Figures 21,22 and 23 show both the images obtained directly from the microscope as well as the result after the image processing steps. All the figures clearly show the substrate bubbles distribution in the reaction

medium, where one could identify the differences in the interfacial area across the scales. Here, the goal was to evaluate the interfacial area for a given time point where a defined substrate conversion is reached, meaning that the reaction time points at which 25% and 50% of substrate conversion were obtained, were different across the reactor volumes. For example, 25% of substrate conversion in the 10 mL reactor did not correspond to the same reaction times as the 20 and 40mL reactors.

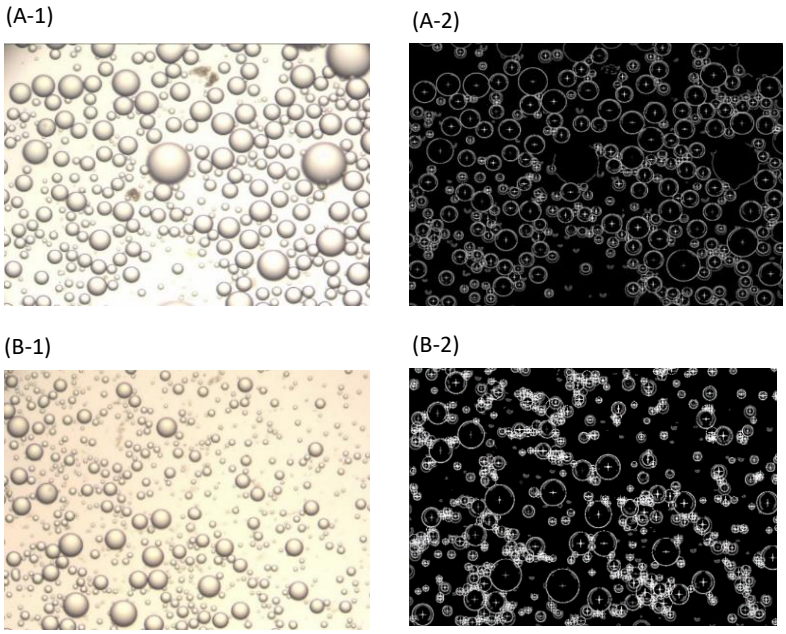


Figure 21 – Images of the biphasic system in the 10 mL reactor observed under the microscope at a 1500x magnification when 25% (A1) and 50% (B1) of MPP conversion was reached. Images on the right-hand side (A2-B2) are processed images with centroid and interfacial area identified.

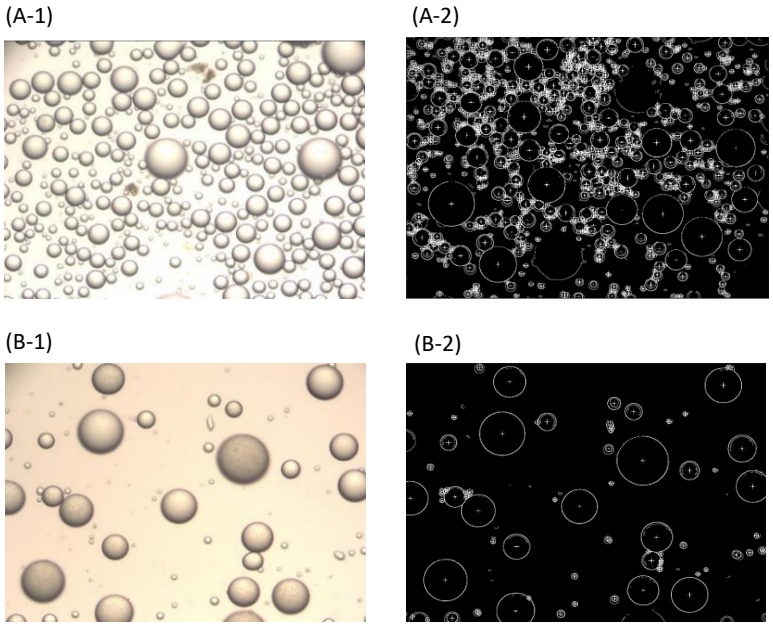


Figure 22 - Images of the biphasic system in the 20 mL reactor observed under the microscope at a 1500x magnification when 25% (A1) and 50% (B1) of MPP conversion was reached. Images on the right-hand side (A2-B2) are processed images with centroid and interfacial area identified.

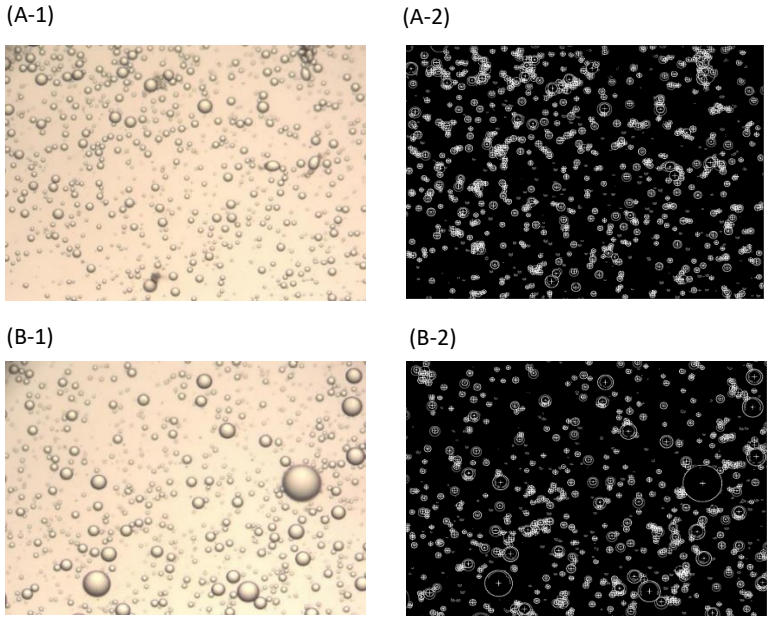


Figure 23 - Images of the biphasic system in the 40 mL reactor observed under the microscope at a 1500x magnification when 25% (A1) and 50% (B1) of MPP conversion was reached. Images on the right-hand side (A2-B2) are processed images with centroid and interfacial area identified.

Figure 21, relative to the 10 mL reactor, appeared to be the one with most substrate bubbles. When looking at Figure 22, there is a difference both in the size and in the quantity of the substrate bubbles between the image related to the time at which 25% and 50% of substrate conversion were reached. The biggest reactor volume was represented in Figure 23, and it seemed that it had the biggest quantity of small radius bubbles. In Figure 22 it was also visible that at 50% of substrate conversion, the bubbles were relatively larger than at 25% of substrate conversion.

Looking at Figures 24, 25, and 26, it can be observed that increasing reactor volume affected the time at which a specific amount of substrate conversion was attained. As the reaction volume increased, so did the time at which 25 and 50% of substrate conversion were obtained. This result corresponds to the results obtained for the reactors mixing efficiency, whereas the scale increased, so did the time that was needed for the channels RGB to stabilize.

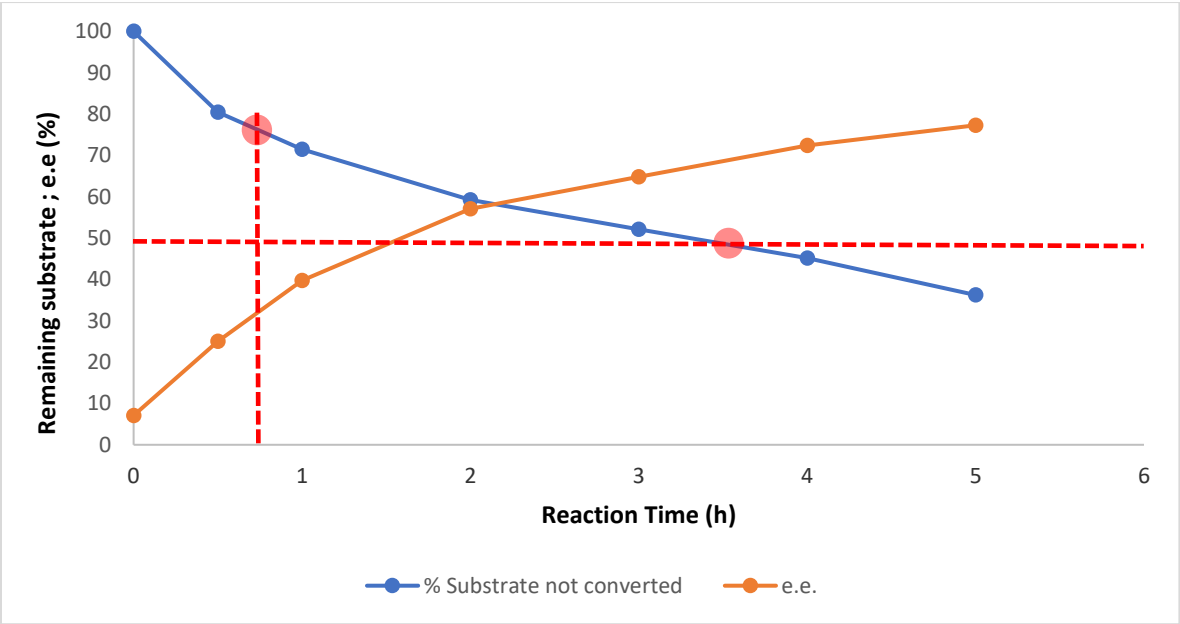


Figure 24 - Remaining substrate and enantiomeric excess of the racemic mixture of MPP at the 10 mL reactor. 25% of substrate conversion was reached after 45min of reaction while 50% of substrate conversion was reached after 3h32min

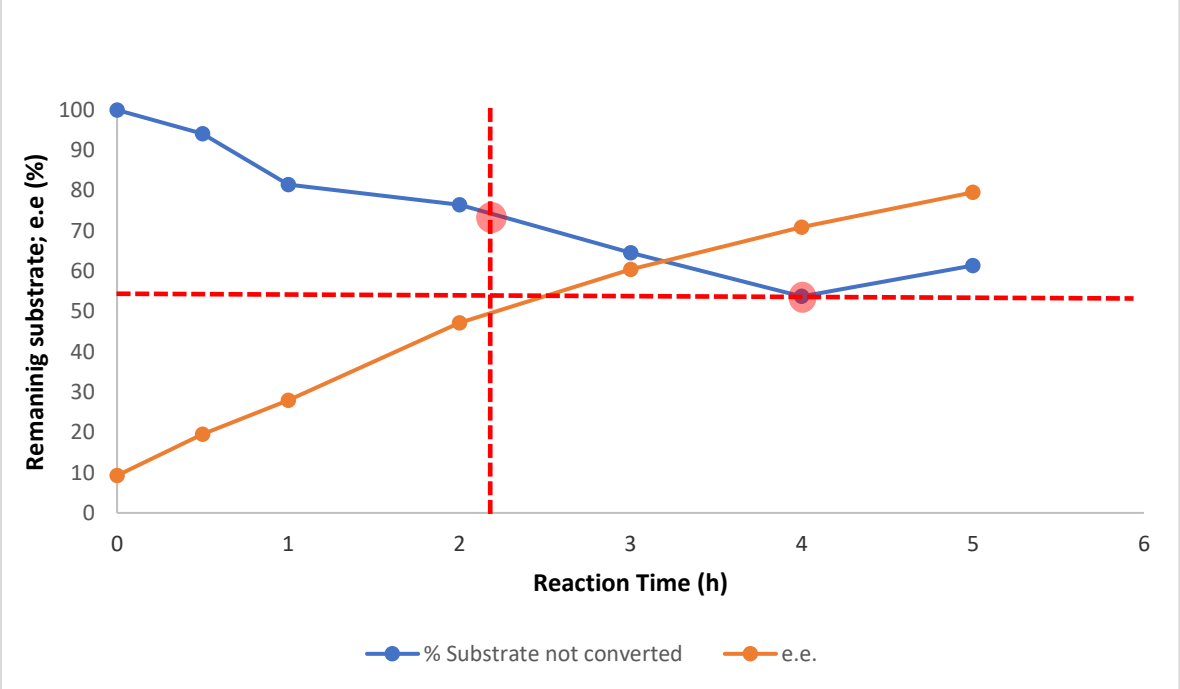


Figure 25 – Remaining substrate rate and enantiomeric excess of the racemic mixture of MPP at the 20 mL reactor. 25% of substrate conversion was reached after 2h08min of reaction while 50% of substrate conversion was reached after 4h

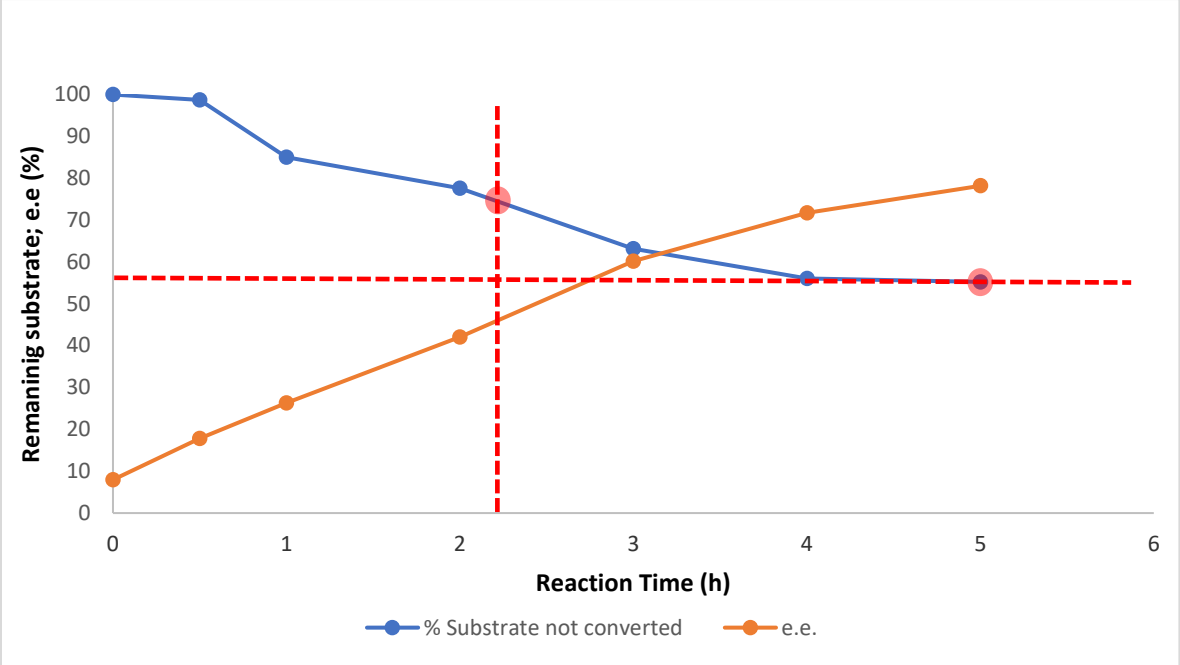


Figure 26 - Remaining substrate and enantiomeric excess of the racemic mixture of MPP at the mL reactor. 25% of substrate conversion was reached after 2h12min of reaction while 50% of substrate conversion was reached after 5h

Figures 27 and 28 represent the bubble radius frequency for a range of substrate bubbles radius across the reactors. The Frequency of the bubble's radius and the related interfacial area for each scale at 25 and 50% can be consulted in the appendix Figures A. 7 – A 11. Looking at Figure 27, 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.

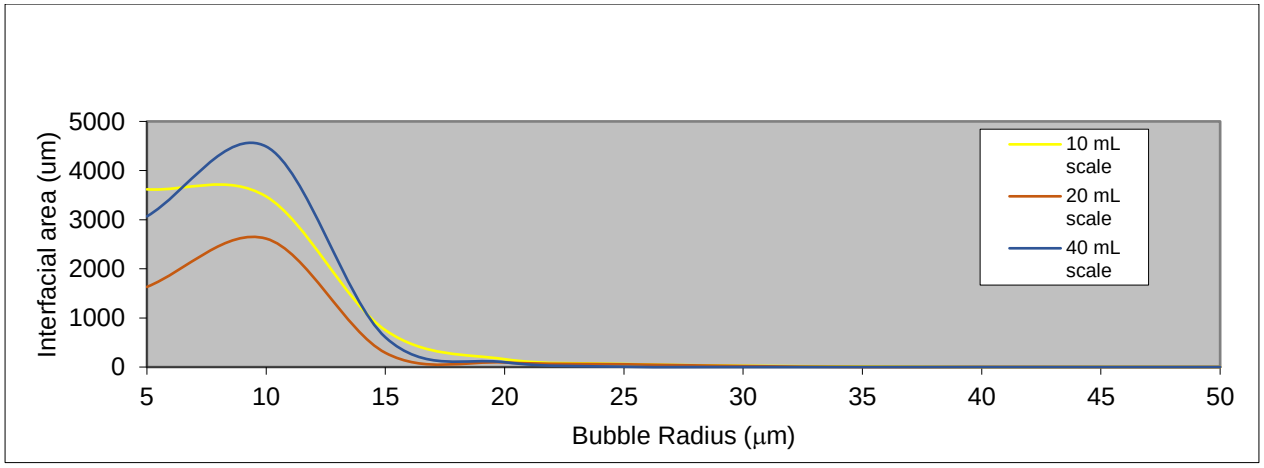


Figure 27 - Bubble radius frequency across the scales when a 25% of substrate conversion was reached

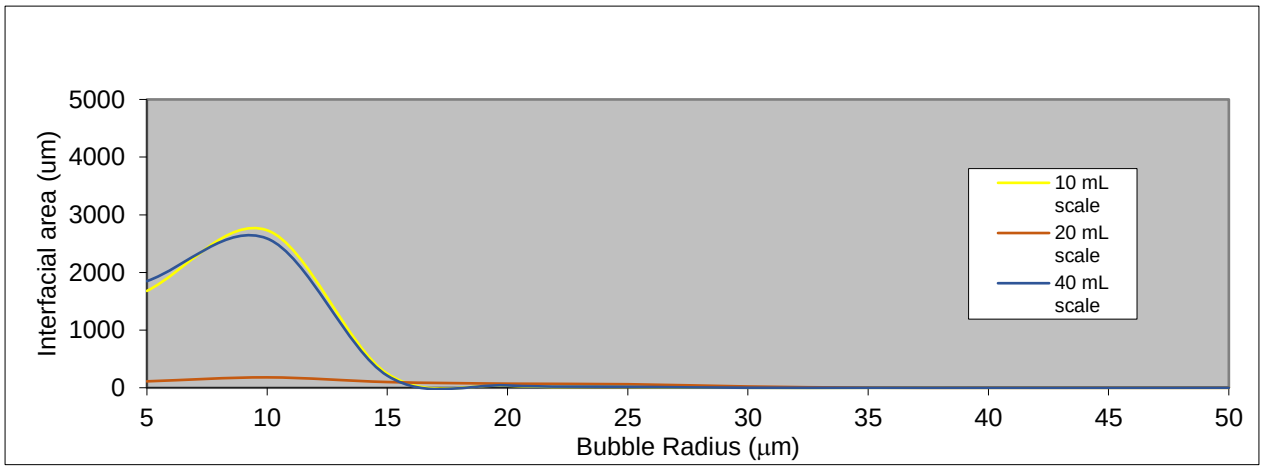


Figure 28 - Bubble radius frequency across the scales when a 50% of substrate conversion was reached

Figure 29 is the representation of the Interfacial area when both 25% and 50% of substrate conversion were reached. When looking at both the figure 29 and Table 21, which is the numerical representation, 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. Observing image Figure 22, which relates to the reaction in 20 mL reactor, it can be observed that it has much fewer substrate bubbles in comparison to the other reactor volumes (figures 21 and 23), which then translates in less interfacial area. This could be caused by experimental errors when extracting samples from the reactor.

Figure 30 represents the activity of EH towards S-methyl-2-phenylpropanoate at a concentration of 40mM in 3 different reactor volumes when at 25 and 50% of substrate conversion, which are the same conditions of the interfacial area study. For the reactions in the 10, 20 and 40 mL reactors, the results follow the trend of figure 29 in the sense that the interfacial area of the reaction decreases with the reaction time, meaning that there is an higher interfacial area when at 25% of substrate conversion in comparison to 50% of substrate conversion. Despite EH loosing activity when the reactor changes from 10 to 20mL (figure 30), which follows the loss of interfacial areal area (figure 29), the same however does not occur on the 40 mL reactor, where the interfacial area appears to be practically the same as in the

10 mL reactor, while the EH activity is significantly lower. These results may present some ambiguity because the reduction of interfacial area could be a possible explanation for less enzymatic activity because less interfacial area results in less mass transfer. However, if one looks at the 40 mL reactor, that rule does not apply, which could mean that the results for this reactor are incorrect, or there are other factors influencing the difference in the enzymatic activity in the scale comparing to the others.

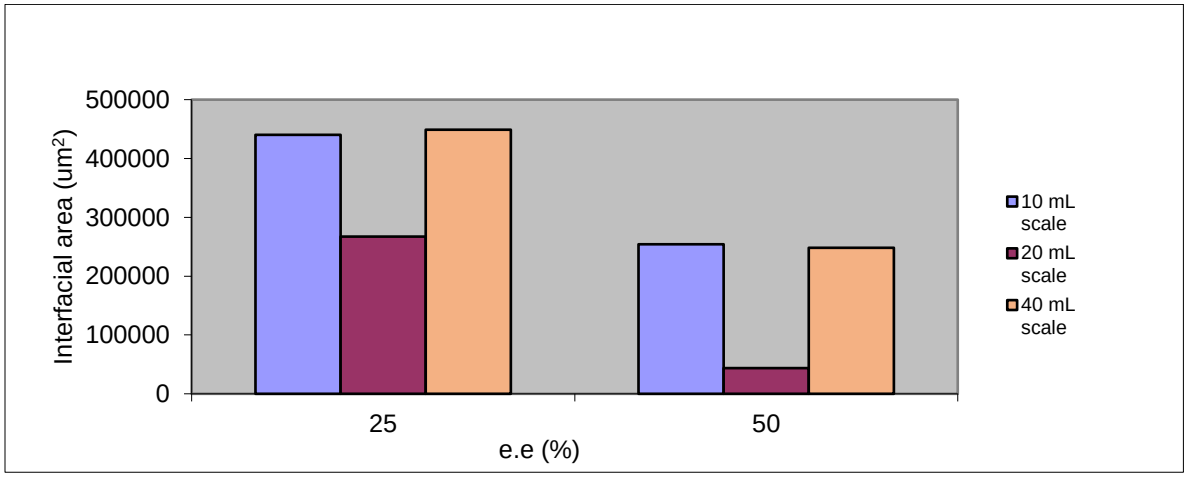


Figure 29 - Interfacial area across the scales when 25% and 50% substrate conversion was reached

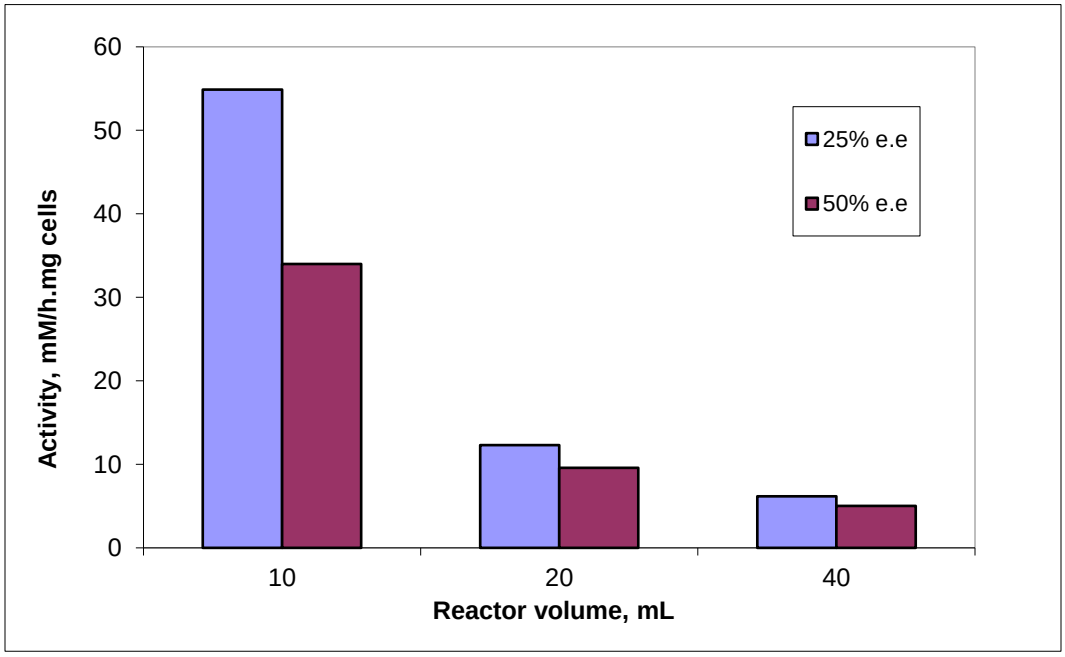


Figure 30 – EH activity towards MPP at 25% and 50% of substrate conversion

Table 21 - Interfacial area across the reactors

	Area (μm^2)		
	25 % substrate conversion	50 % substrate conversion	Total
10 mL reactor	440199.96 μm^2	254123.43 μm^2	694323.39 μm^2
20 mL reactor	267223.87 μm^2	43730.97 μm^2	310954.84 μm^2
40 mL reactor	448839.34 μm^2	248405.73 μm^2	697245.07 μm^2

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 conversion were attained during the reaction course. The largest 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 μm, 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[114] 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 increases. 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 [115] 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 (Figure 29), 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, a larger interfacial promotes a better distribution and a higher activity in a biphasic system [116]. 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 [117]. 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.

3.5. Substrate concentration and reactor volume effect 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 took part in the INMARE project [61] 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 was assessed 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 [118] 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 22. The following figures 31, 32 and 33, relate to the enzymatic activity towards different substrate concentrations (40,80 and 160mM) at different reactors (10,20 and 40mL).

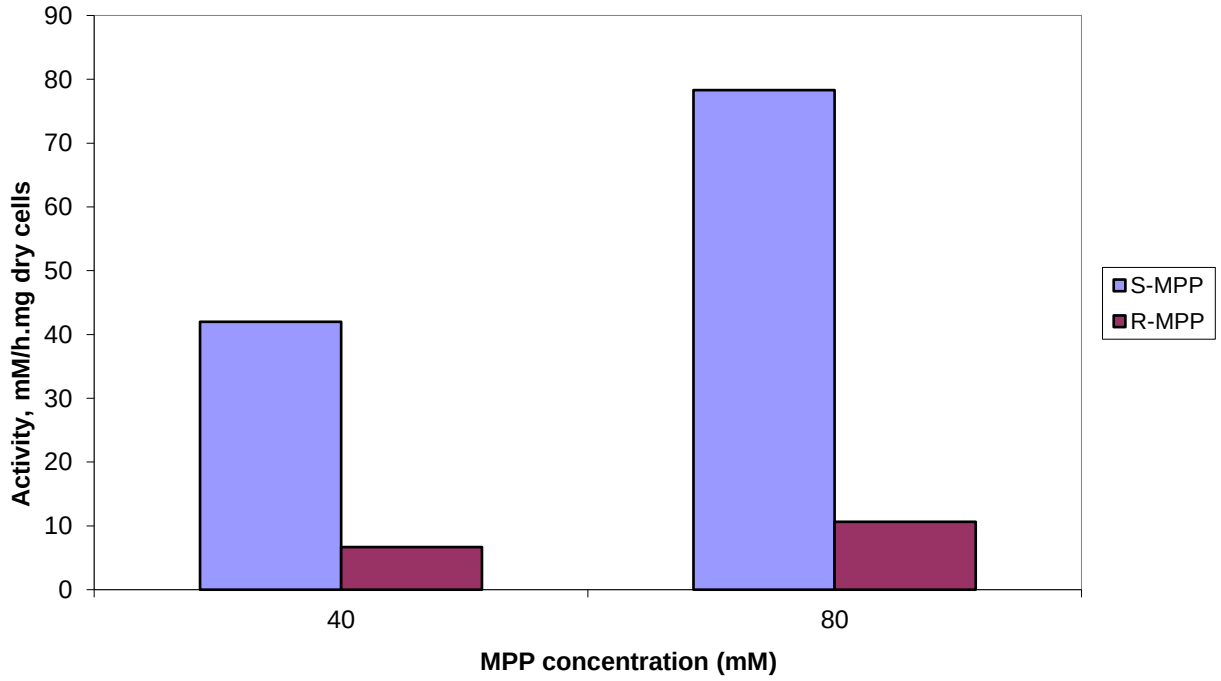


Figure 31 - Enantioselective hydrolysis of methyl-2-phenylpropanoate (40mM) with lyophilized EH cells during 5 hours of reaction at the 10 mL reactor.

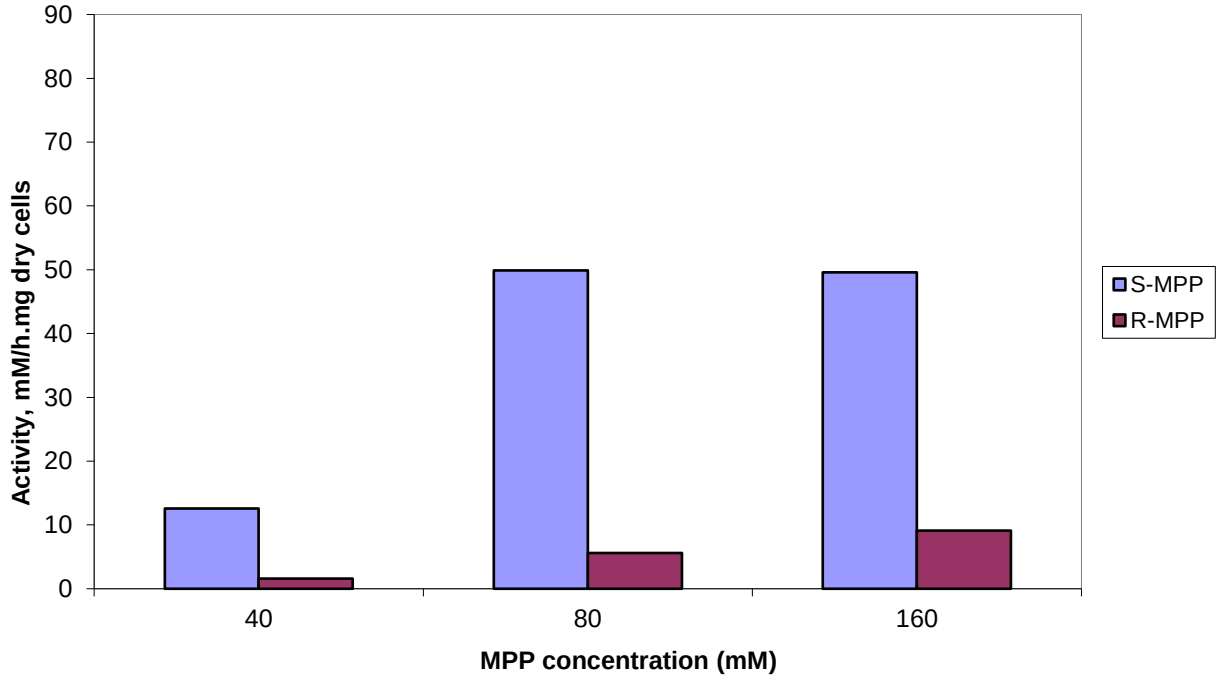


Figure 32 - Enantioselective hydrolysis of methyl-2-phenylpropanoate (40mM) with lyophilized EH cells during 5h (40mM) and 6h (80 and 160 mM) of reaction at the 20 mL reactor.

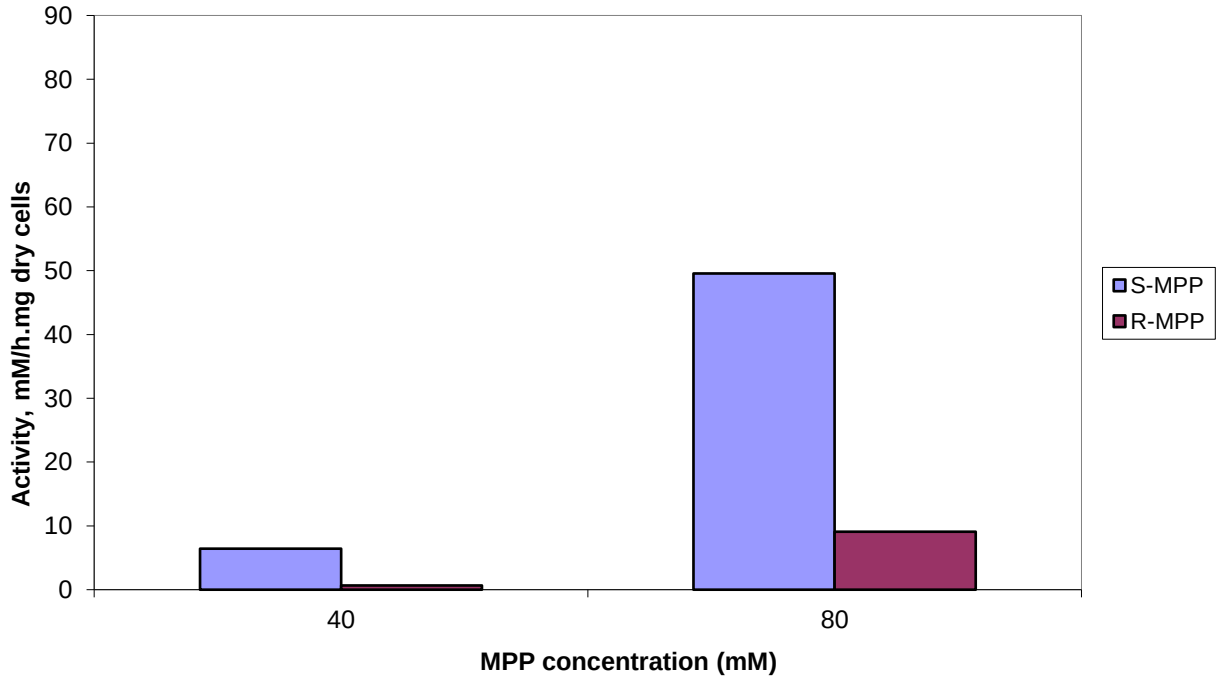


Figure 33 - Enantioselective hydrolysis of methyl-2-phenylpropanoate (40Mm) with lyophilized EH cells during 5h (mM) and 6h (80 mM) of reaction at the 40 mL reactor.

Figures 34 and 35 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.

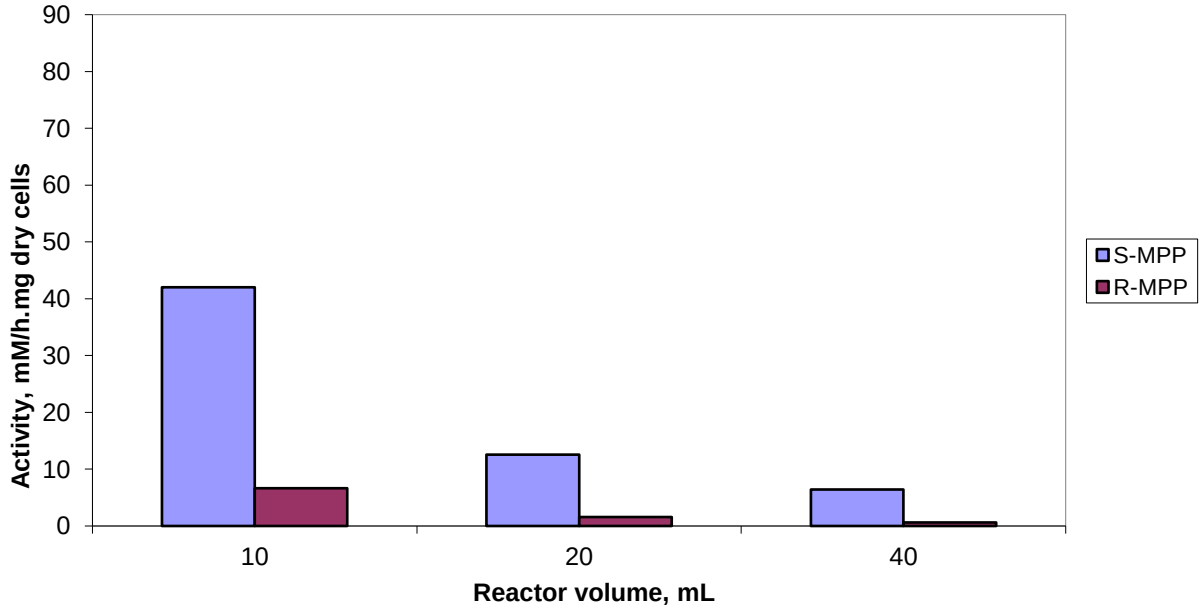


Figure 34 - Enantioselective hydrolysis of methyl-2-phenylpropanoate (40Mm) with lyophilized EH cells during 5 hours of reaction for all reactor volumes.

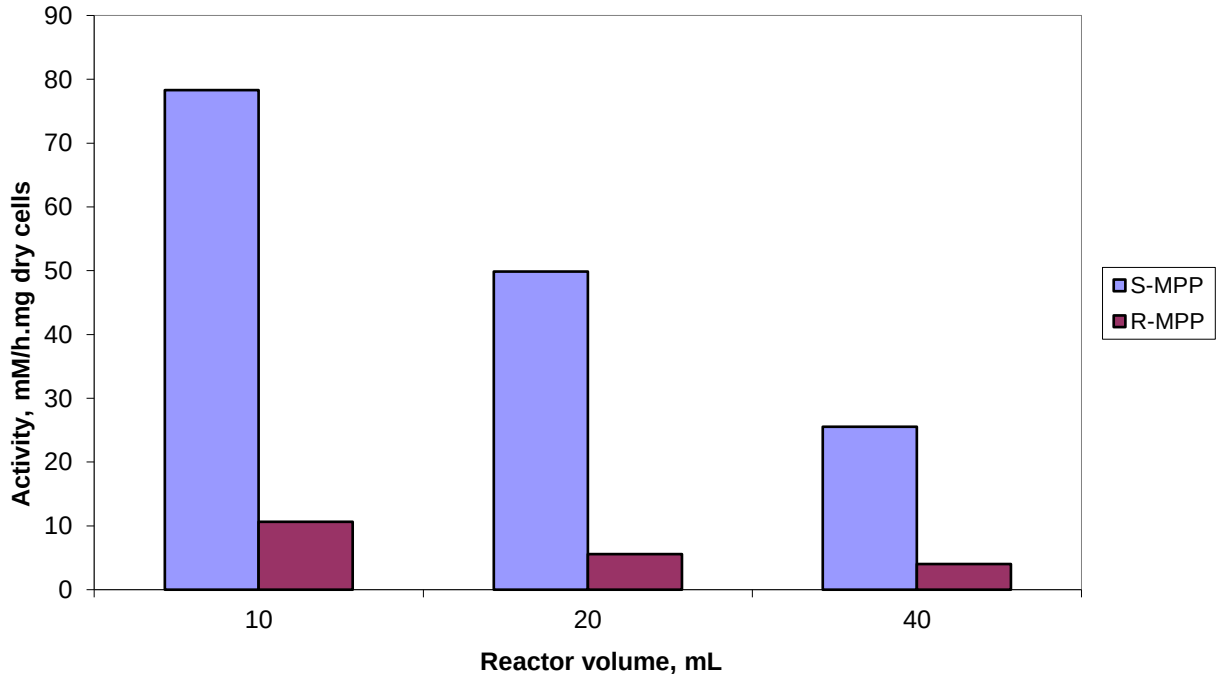


Figure 35 - Enantioselective hydrolysis of methyl-2-phenylpropanoate (80Mm) with lyophilized EH cells during 6 hours of reaction for all reactor volumes.

Table 22 - Results of the enantioselective hydrolysis of methyl-2-phelylpropanoate with the lyophilized EH cells.

Reactor Volume	[MPP], mM	Activity towards S-MPP (mM/h)	Activity towards R-MPP (mM/h)	Activity towards S-MPP (mM/h. mg dry cells)	Activity towards R-MPP (mM/h. mg dry cells)	E.E (%)	% Converted substrate
10	40	42.00	6.66	8.82	1.40	77.30	63.77
	80	78.28	10.63	16.44	2.23	49.01	31.48
20	40	12.55	1.59	5.27	0.67	79.56	38.61
	80	49.88	5.60	20.95	2.35	65.60	56.48
	160	49.58	9.11	20.83	3.83	33.47	26.09
40	40	6.43	0.65	21.46	3.38	78.19	44.76
	80	25.55	4.02	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 mixing time 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 was lower in the bigger reactor, 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 22, 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 [118], 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 at a small scale.

4. Conclusions and Future Work

The developed work involved the evaluation of 2 biocatalysts, EH and Abo in terms of their activity and enantioselectivity toward 2 substrates, MPP and *p*-NPB for different reactor volumes, also assessing important reactor characteristics such as their mixing efficiency. 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.

5. References

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6. Appendix

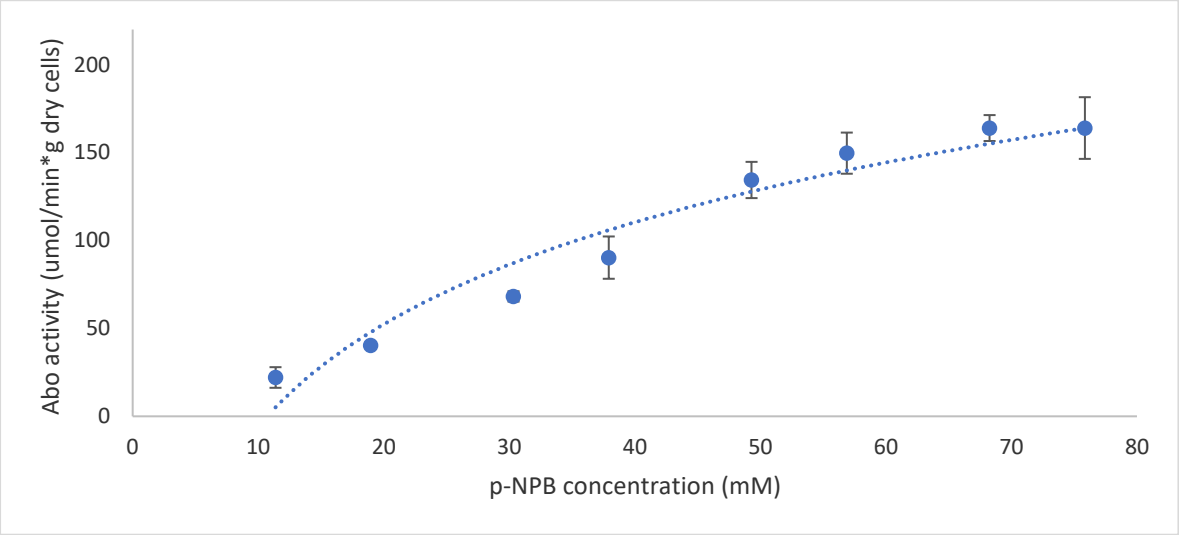

Figure A. 1 - Michaelis-Menten Kinetics for the activity of Abo activity towards the substrate p-NPB

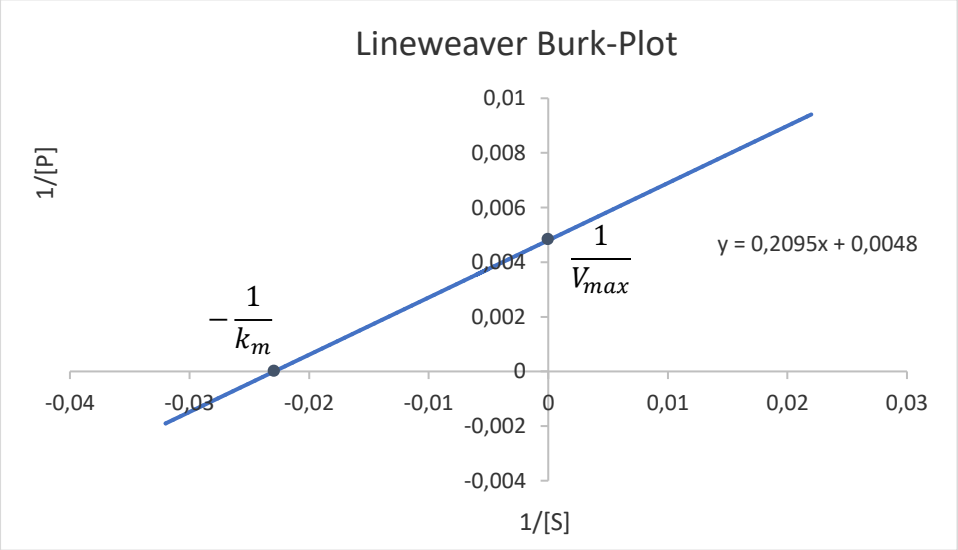

Figure A. 2 - Lineweaver Burk-Plot for the activity of Abo activity towards the substrate p-NPB

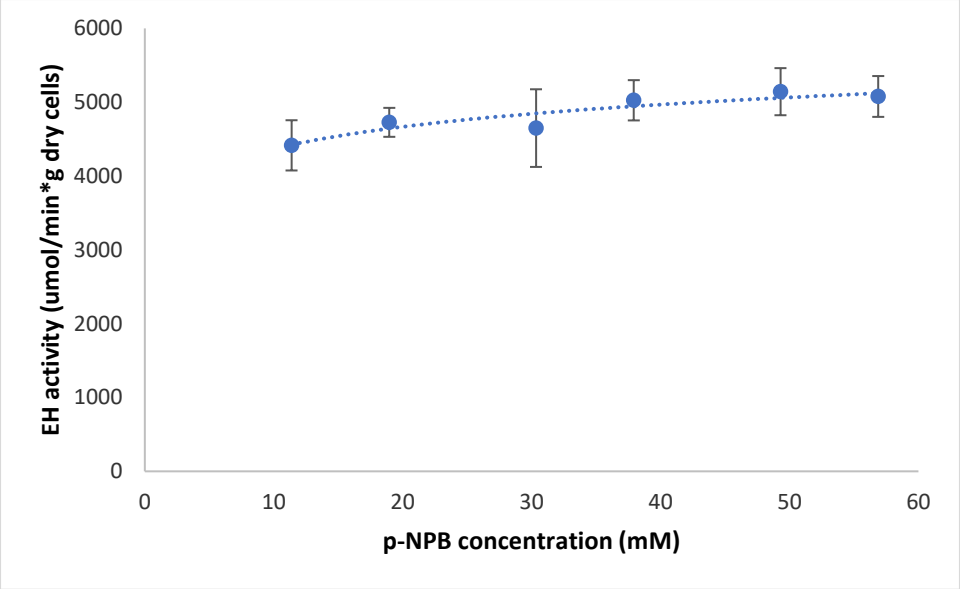

Figure A. 3 - EH activity towards p-NPB (does not follow Michelis-Menten kinetics for the selected enzyme and substrate concentration)

RGB workflow analysis on image J – Macro:

- File >> Import >> Image Sequence >> Select image >> Ok; Use Virtual Stack
- Image >> Color >> Make Composite
- Image >> Color >> Split channels
- Analyze >> Tools >> ROI Manager >> select 3 regions to measure >> Select all ROI added >> More >> Multi measure
- Copy data to excel

Interfacial area analysis on FIJI – Image J Full Macro:

```
// Binarize images

run("8-bit")

bin = 2;

//Make background intensity uniform

i=getTitle();

run("Median...", "radius=2"); //Denoise

run("Duplicate...", "title=2");

selectWindow("2");
```

```
run("Gaussian Blur...", "sigma=80");

run("Invert");

imageCalculator("Add", i,"2");

close("2");


//Run low pass to get rid of high frequency noise

run("Gaussian Blur...", "sigma=3");


//Create edge image

run("Find Edges");

setAutoThreshold("Default dark");

setOption("BlackBackground", false);

run("Convert to Mask");


//Skeletonize to thin out edges and suppress non circle edges

run("Skeletonize");

run("Grays");


//Increase edge thickness so large cicles can be found

for(a=1; a<=4/bin; a++) run("Erode");

run("Bin...", "x=" + bin + " y=" + bin + " bin=Max");


//Run Hough Circle Transform

run("Hough Circle Transform","minRadius=" + round(10/bin) + ", maxRadius=" + round(400/bin) + ", inc=1,
minCircles=1, maxCircles=65535, threshold=0.6, resolution=904, ratio=1.0, bandwidth=10, local_radius=10,  reduce
show_mask show_scores results_table");
```

Apply Macro to all images (Hough Circle Transform):

Process >> Batch >> Macro;

Input: Original Images

Output: Images with Hough Circle transform

Macro: Hough Circle Transform

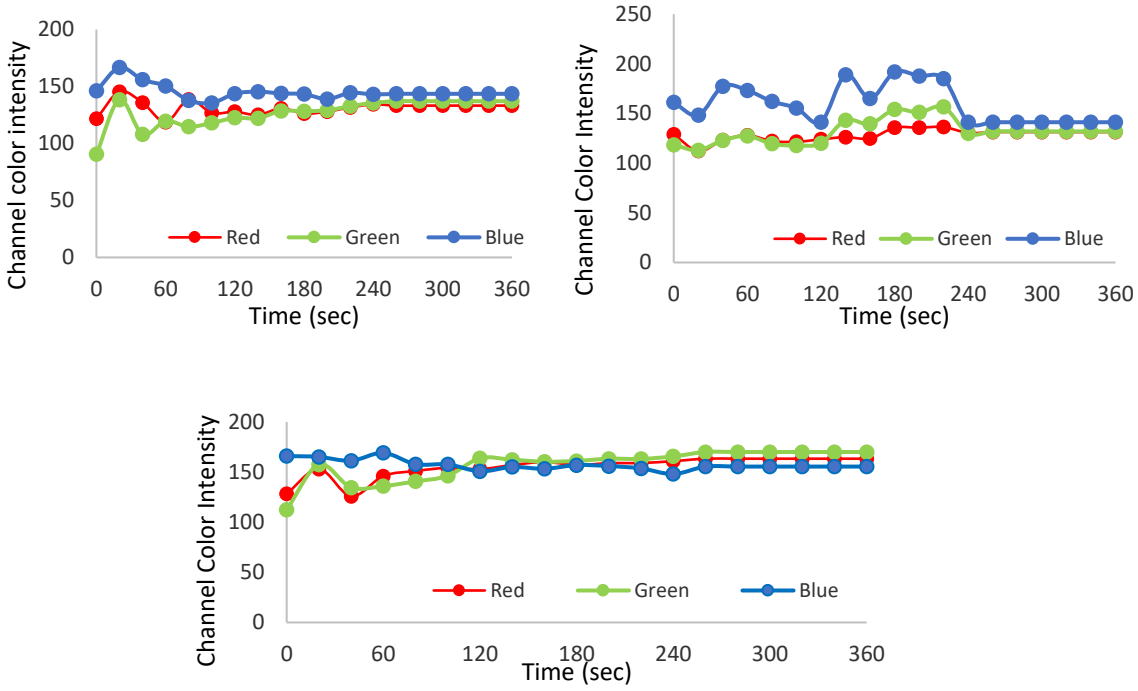

Figure A. 4 - Colour Intensity change (RGB channels) over time for 3 selected pixelized areas in the 10mL scale reactor

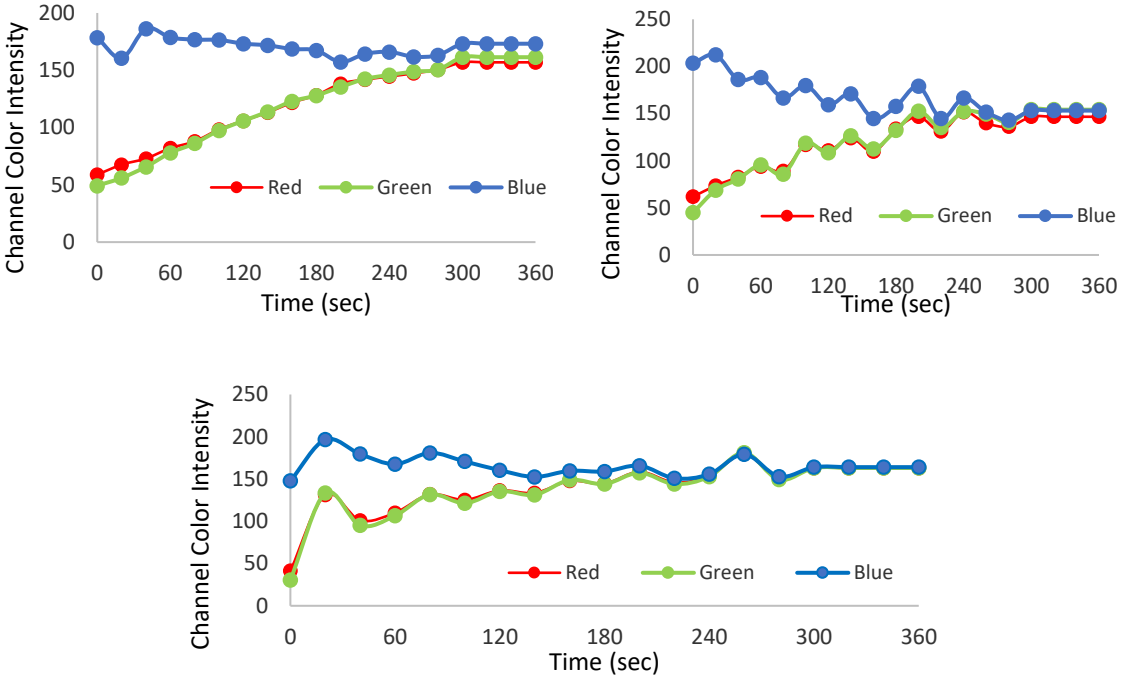

Figure A. 5 - Colour Intensity change (RGB channels) over time for 3 selected pixelized areas in the 20mL scale reactor

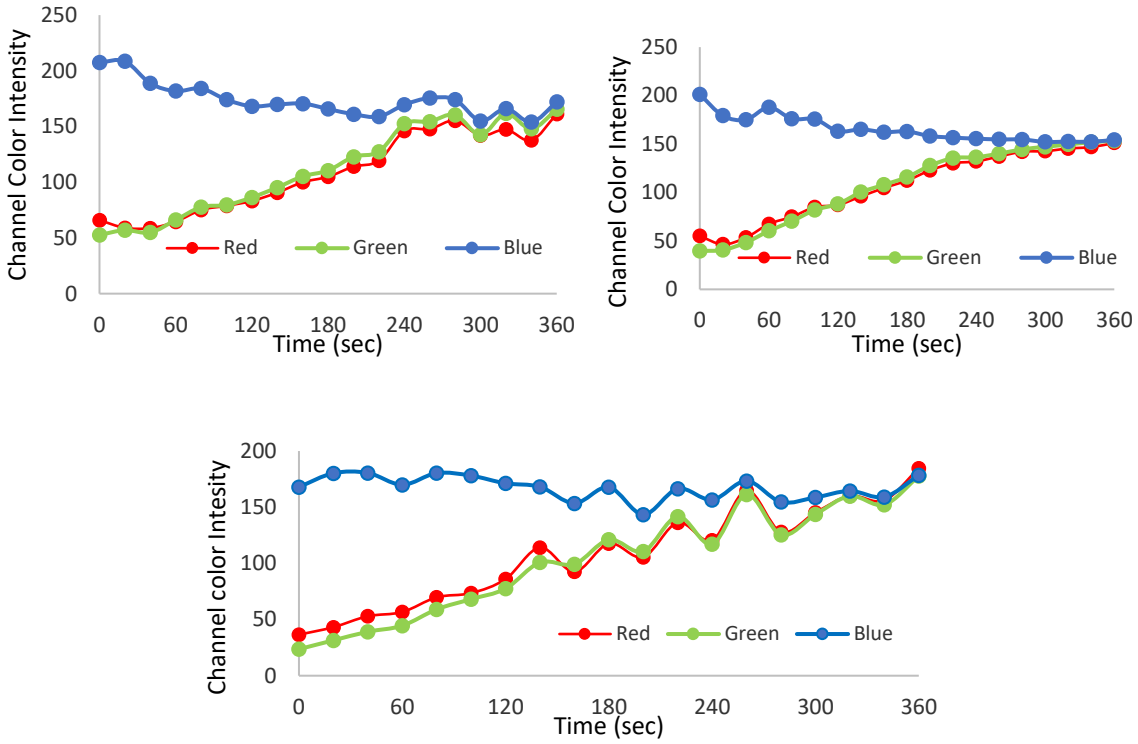

Figure A. 6 - Colour Intensity change (RGB channels) over time for 3 selected pixelized areas in the 40mL scale reactor

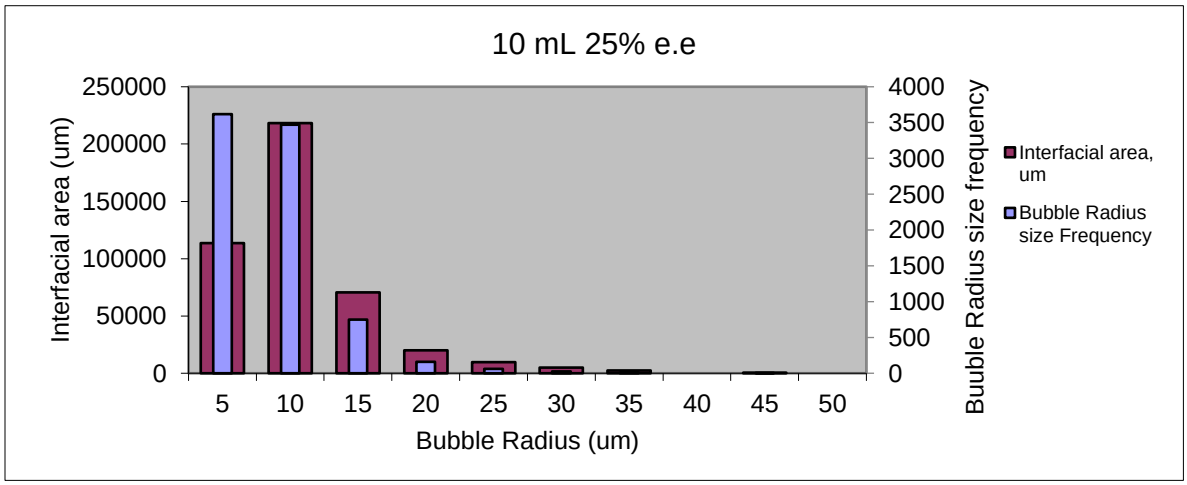

Figure A. 7 - Interfacial area (um) and bubble Radius Frequency for 10 mL scale at 25% of substrate conversion

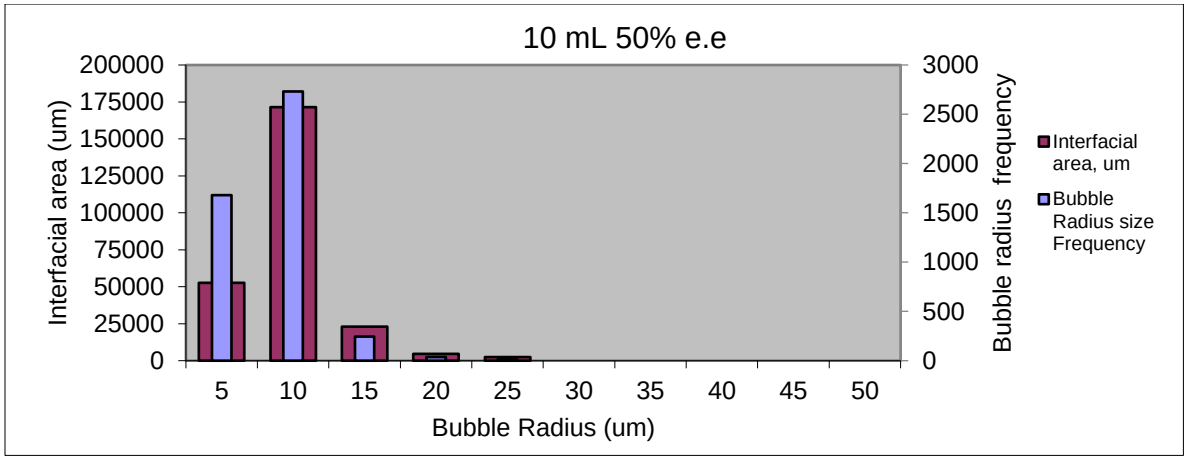

Figure A. 8 - Interfacial area (um) and bubble Radius Frequency for 10 mL scale at 50% of substrate conversion

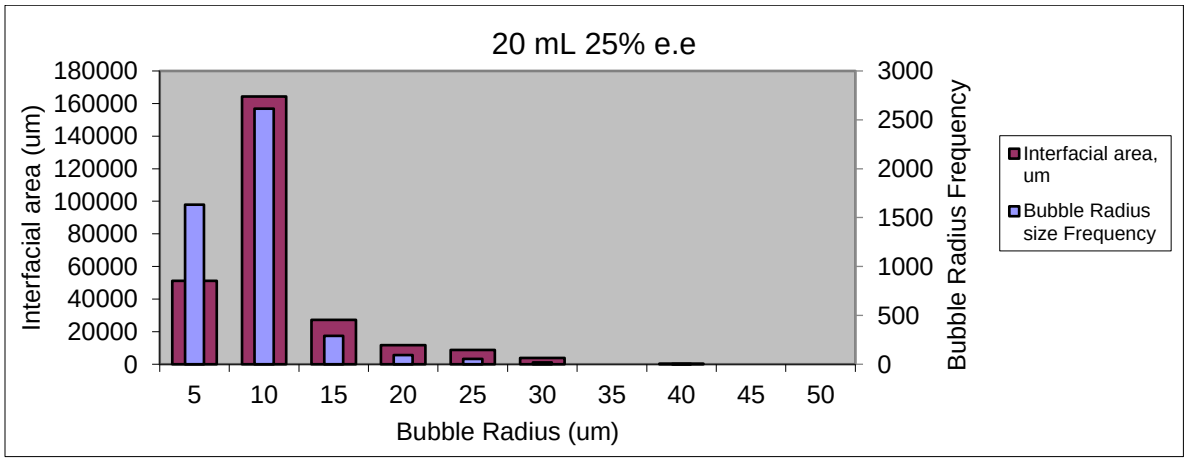

Figure A. 9 - Interfacial area (um) and bubble Radius Frequency for 20 mL scale at 25% of substrate conversion

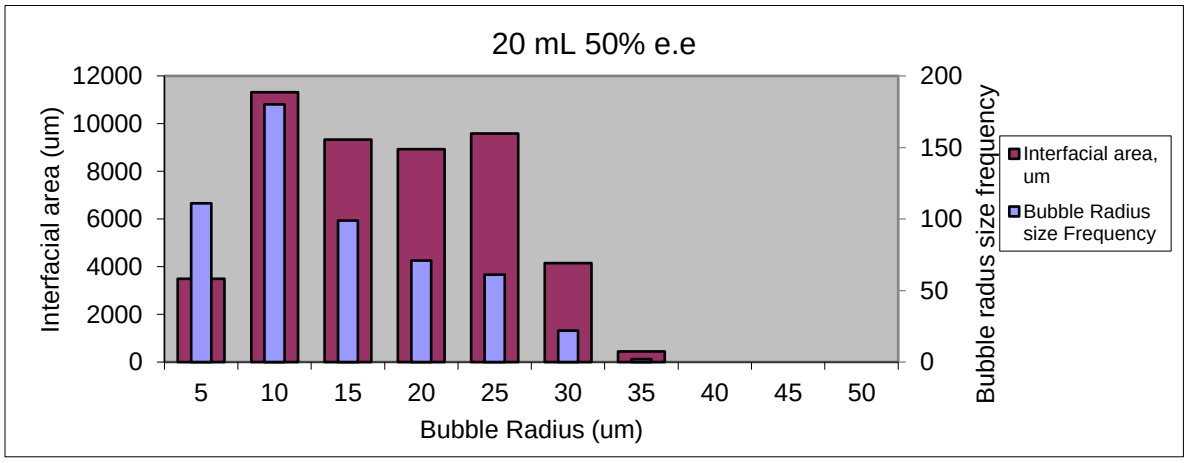

Figure A-9 - Interfacial area (um) and bubble Radius Frequency for 20 mL scale at 50% of substrate conversion

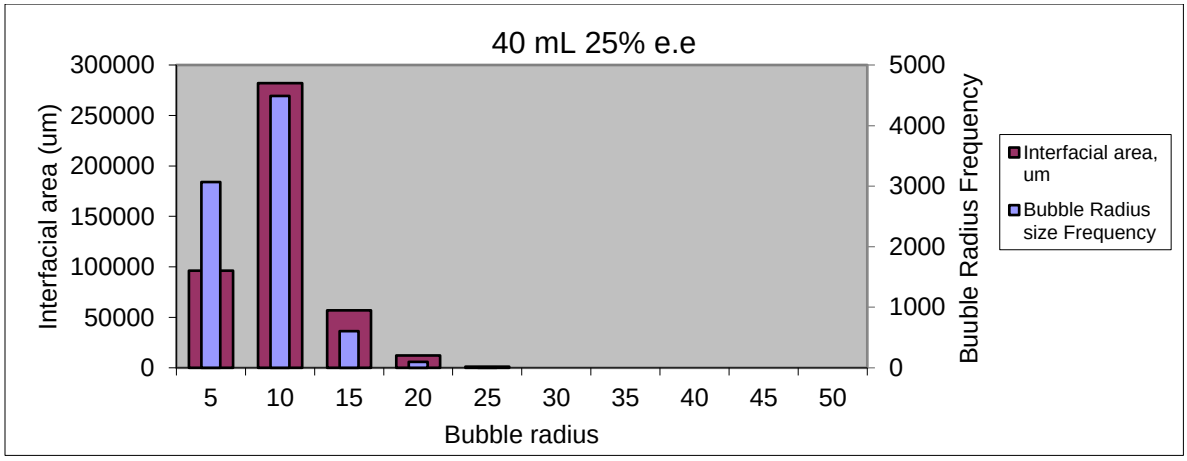

Figure A. 10 - Interfacial area (um) and bubble Radius Frequency for 40 mL scale at 25% of substrate conversion

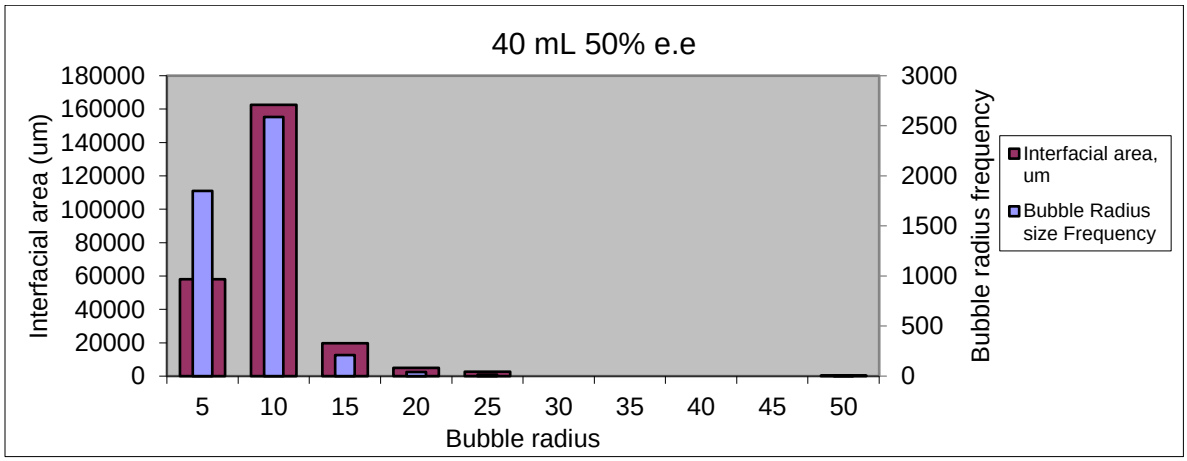

Figure A. 11 - Interfacial area (um) and bubble Radius Frequency for 40 mL scale at 50% of substrate conversion