Master Thesis

Evaluation and degradation of microplastics

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Abstract: Currently, the demand for plastic materials is increasing, leading to an increase in waste generation, which favors the emergence of a new type of micropollutant called microplastics (MP), which affect living beings due to the effects of bioaccumulation and biomagnification. This project evaluates the enzymatic method for the degradation of PET, mainly derived from containers and packaging products. This work explains how to properly design the necessary tests in order to assess the effectiveness of hydrolytic enzymes in the degradation of MP. To this end, enzyme activity is monitored using spectrophotometric techniques considering Michaellis-Menten enzyme kinetics in different buffer media. Moreover, PET morphology is studied to determine how these enzymes affect this plastic substrate. The results show a reduction in the size of the particles found, as well as an increase in their quantity under optimal enzyme conditions for pH and temperature. A variation in enzyme activity is observed depending on the buffer medium used. The main conclusion is that enzymes aid in the degradation of MP under suitable environmental conditions, primarily by reducing their size. Enzymatic activity can also be affected by ionic strength of the medium where the reaction occurs, which should be considered for further studies due to possible enzyme inhibition.

Keywords: Microplastics; biodegradation; enzymes; plastic pollution; PET degradation; enzyme kinetics; Michaellis-Menten

1. Introduction

Most plastics are made from products derived from petroleum, natural gas, and coal. These plastics include polyethylene terephthalate (PET), high-density polyethylene (HDPE), low-density polyethylene (LDPE), polypropylene (PP), and polyvinyl chloride (PVC). These materials primarily exhibit the following properties: light weight, heat resistance, high malleability, good toughness, and high tensile strength. These properties have allowed them to be used in a wide variety of applications, such as telephonic devices, radios, plumbing pipes, synthetic clothing, personal items, and infrastructure elements, among others. They are currently very important in a wide range of industries, including textiles (sportswear), automotive (bumper), packaging (water bottles) and electronics (cable coating)[1], [2].

This wide variety of applications for plastics has caused the demand for this resource to increase from 2 million tons in 1950 to approximately 400 million tons in 2018. It is estimated that around 8 million tons of plastic are dumped into the sea annually, affecting marine life and, through food chains, also terrestrial life [3]. This accumulation favors the appearance of new types of micropollutants called microplastics (MP), which, with a size less than 5 mm, have various harmful effects on organisms.

The European Parliament has reported on its website the presence of approximately 51 billion of these types of MP particles in the sea [3]. This high presence of MP particles is causing them to be consumed by various organisms in the marine environment, causing a decline in their proliferation. This is due not only to reduced access to nutrients, which are replaced by these MP in their diet, but also to their satiating effect and interaction with their reproductive system. Once inside the organism, these particles can be retained by it (bioaccumulation) or pass to higher levels of the food chain (biomagnification). These harmful effects can be enhanced by the ability of MP

to absorb other contaminants such as zinc or copper, increasing the adverse effects on organisms [4].

There are various treatments for reducing plastic waste. The main recycling treatments can be classified as: 1) Physical: landfills or incineration; 2) Chemical: hydrolysis, alcoholysis, or glycolysis; and 3) Mechanical: crushing, grinding, compaction or pressing, separation, extrusion, and delamination, among others. Most of these treatments have subsequent environmental impacts due to the release of secondary pollutants (generated in the environment due to reactions between primary pollutants and atmospheric elements) and hazardous byproducts (such as dioxins, toxic metals, and furans generated in industrial processes). Mechanical recycling would be a valid option for reducing plastic waste without producing polluting byproducts, but it is currently quite inefficient because this kind of pollutant arrives mixed with impurities, and with each recycling cycle, the polymer loses mechanical properties such as viscosity [2].

A new treatment method, biological treatment, has recently emerged, which has good prospects and a low environmental impact. The biological method focuses on the isolation and study of hydrolytic enzymes that break the bonds present in the structure of plastics, coming from bacteria and fungi. In this project, the effectiveness of this new degradation method is studied, as well as its potential in the destruction of MP bonds, focusing on polyethylene terephthalate (PET) type. This work mainly analyzes the degradation of PET due to its high presence in the plastics currently used with almost 70 million tons annually [5], being mainly used for packaging in the food sector. Enzymes cable of hydrolyzing PET includes PETase, MHETase, lipases, cutinases, and carboxylesterases, which, by degrading PET, allow the original monomers, terephthalic acid (TPA) and ethylene glycol (EG), to be obtained, facilitating their recovery as raw materials and the circular economy. Hence, this treatment has good prospects especially highlighting the enzymes PETase and MHETase [2].

Plastic pollution is one of today's most significant environmental problems, due to the wide variety of synthetic materials used in everyday items such as food packaging, the increasing demand for these materials, and the rise in plastic waste generated. This master's Thesis aims to analyze the effectiveness of these plastics in degrading and reducing pollution using enzymes, an environmentally friendly method that does not generate residual pollution or secondary contaminants. This project will demonstrate the techniques required to characterize hydrolytic enzymes and monitor the enzymatic degradation of PET, as well as provide a laboratory-scale economic perspective on the potential cost of this biochemical method for degrading these micropollutants.

2. State of the Art

2.1. Conventional treatment of PET waste

Currently, most plastic waste is disposed of through physical or thermal treatments, such as landfill disposal or incineration, or through chemical treatments that convert PET into lower molecular weight products. These waste disposal methods present several environmental disadvantages due to the release of harmful pollutants, promoting climate change, and the creation of toxic byproducts that affect health [2].

Due to the environmental impacts, mechanical recycling is being used, a sustainable alternative that does not generate MP during the process but is very inefficient and can generate poorly recycled products due to the appearance of mechanical stresses in the grinding, extrusion, etc… processes. For this reason, new methods of plastic degradation are currently being sought using an enzymatic method, although it is still under investigation [2]. This new method is developed in greater depth in the following section.

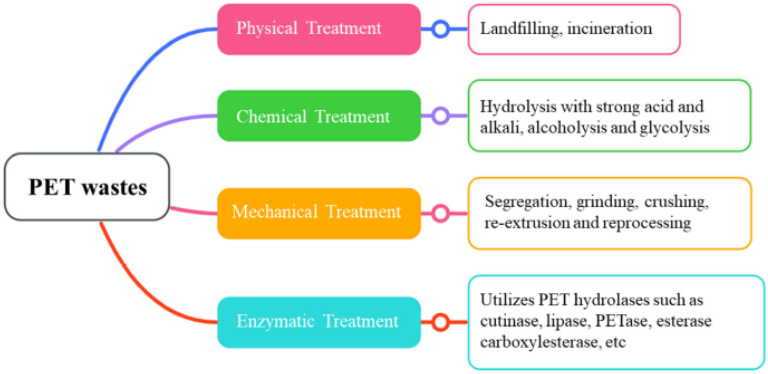


Figure 1: Types of PET waste treatments ***[2]***

2.2. Biological treatment of PET waste

In recent years, and in response to the increasing generation of plastic waste, more efficient and environmentally friendly alternatives than those mentioned in the previous section have been sought. It has been discovered that certain microorganisms generate a type of hydrolytic enzyme capable of breaking the ester bonds that join PET monomers. The most common strategy for finding these microorganisms is collecting samples from areas rich in plastic waste and cultivate them in media with the plastic to be degraded. The growth of the organisms will determine their degradative capacity. In recent years, samples are being analyzed based on their metagenome (set of genes of the microorganisms in each ecosystem) [6].

Table 1: Microorganisms that degrade plastics ***[6]***

Microorganisms	Plastic
Ideonella sakaiensis	PET
Thermobifida fusca	PET, PHA, PCL, PL
Aspergillus nidulans	PET, PCL, PL, PBS
Pseudomonas sutzeri	PP
Alicycliphilus BQ1	PU
Cochliobolus sp.	PVC
Rhodococcus ruber	PS

Since this project considered PET samples, it will delve into enzymes capable of breaking the bonds that make up this type of plastic. These enzymes typically degrade with the help of water molecules, which has allowed us to identify a wide variety of enzymes with a high potential to degrade PET bonds, although in most cases, their degradation efficiency has not been high.

Currently, 24 different PET hydrolytic enzymes (PHE) have been identified. These enzymes belong to the EC 3.1 class of lipases, cutinases, and esterases, which are characterized by acting on ester bonds. These enzymes usually have one or two disulfide bridges (important for the integrity and stability of the enzyme), the same conserved catalytic domain (a catalytic triad consisting of a serine, a histidine, and a negatively charged residue that can be an aspartate or glutamate, which work together in the active site to break the ester bond), and a typical alpha/beta folding (a distinctive three-dimensional structure of enzymes) [7].

When PET is reacted with a hydrolase, the products obtained are EG and TPA, both of which are monomers that do not affect the environment. Additionally, BHET and mono-(2-hydroxyethyl) (MHET) are also produced, which are transient hydrolysis products [7]. The following image shows the PET degradation scheme:

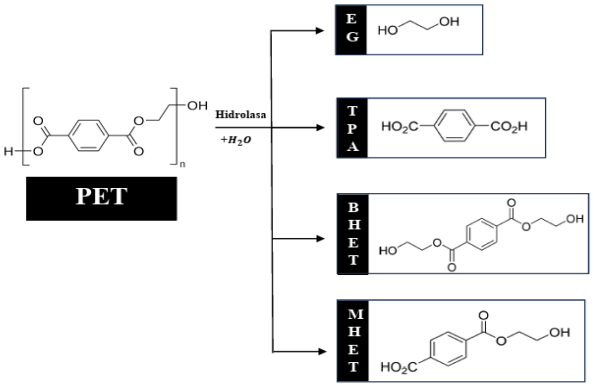


Figure 2: Chemical structure of PET and its degradation products ***[7]***

The use of hydrolytic enzymes is aligned with the sustainable circular economy model for PET. An enzymatic bioreactor composed of PET hydrolases and carboxyl esterases has been shown to facilitate recycling using this enzymatic method, being efficient in hydrolyzing the amorphous portion of PET into its TPA and EG monomers, which can be recovered through precipitation and distillation for reuse in PET synthesis. The disadvantage of these processes is the high energy requirements, making them impractical [7].

Currently, in the industrial sector, Carbios and Novozymes are promoting the large-scale deployment of enzymatic PET degradation technology through the construction of a recycling plant in the Longlaville region of France. This plant is expected to open in 2025 and will have a processing capacity of 50,000 tons of waste per year. It is the first plant in the world to integrate the technology for biological PET recycling[8].

3. Objectives

The following objectives have been established for this Master´s Thesis:

- Several enzymes that can be used for MP degradation will be investigated, focusing on those currently on the market. To this end, the properties of these enzymes will be studied and validate their effectiveness in breaking bonds in the laboratory. In parallel, databases of these enzymes will be analyzed, as well as the commercial websites where can be obtained.
- Relevant studies will be conducted to compare some of the hydrolase enzymes currently on the market, assessing their efficiency in breaking down the chemical bonds that make up MPs using the Michaelis-Menten kinetic-enzymatic model. The performance of the enzymes in MP degradation will be compared, and conclusions will be drawn for future research on the use of enzymes in MP degradation.
- The necessary use of laboratory equipment, techniques, and tools to preserve hydrolase enzymes in good condition will be technically described, including the application of these enzymes to water samples and their efficiency, as well as the preparation of the media in which the tests will be performed. Also, it will be learnt how to use various software programs that will allow the evaluation of the results of the application of different instrumental techniques aimed at analyzing the destruction of microplastics.
- An economic analysis and estimation will be performed at the scale laboratory for the use and treatment of commercially available enzymes in the micropollutant removal processes in each of the different water samples being tested. This estimation will consider the price of the various elements, tools, and technologies involved in enzymatic degradation processes in the laboratory.

4. Methodology

This project has been divided into three main phases. The first phase is research, while the second and third are experimental phases:

4.1. Research and compound selection

Research will be conducted to understand the functioning of enzymes and what types of enzymes can serve the purpose of this project. In this phase, it will primarily use scientific articles and publications and databases such as Uniprot.org [9]and Expasy.org[10], to study the characteristics of these enzymes. This research phase will focus primarily on the commercial enzymes currently distributed by various distributors. Furthermore, using these documents and database programs, properties of the enzymes found will be analyzed, as well as their operating conditions in relation to pH and temperature. For this project, two types of enzymes (lipases and cutinases) that can meet the objective of degrading the amount of MP in the environment will be considered:

- Lipase B from *Candida antartica* (EC 3.1.1.3) is being used to catalyze various reactions such as hydrolysis, esterification, ammonolysis, and C-C bond formation. This is due to its excellent catalytic properties, such as high selectivity, stability in both organic solvents and thermally, enantioselectivity (the preference of a chemical reaction to form one enantiomer over its mirror image), and the ability to function without cofactors. This enzyme is currently being used in pharmaceutical, chemical, food, and energy industries [11][12].The range of enzymatic activity for this enzyme is quite high, ping pH operating ranges from 6-8.5 (with a maximum at 7.5) and temperature ranges from 20-70 ºC, presenting its maximum at 55 ºC.
- Cutinase 2P from the fungal organism *Arxula Adeninivorans* (EC 3.1.1.74) which is widely used for industrial biotechnology. This enzyme, also called ACUT2 in the scientific article [13], corresponds to a certain DNA sequence that encodes a cutinase gene allowing enzymatic action on natural cutins, biodegradable synthetic polyesters and model substrates such as paranitrophenol butyrate. The range of enzymatic activity for this enzyme is quite high, having pH operating ranges from 3.5-8 and temperature ranges from 5-60 ºC, presenting its maximum at 37 ºC.

4.2. Definition of enzymatic activity

Once the enzymes are selected, kinetic parameters of these enzymes are analyzed, making use of the Michaelis-Menten model and the Lambert-Beer Law. For this purpose, a reference substrate, paranitrophenol butyrate (also called pNPB, is a substrate that allows defining the catalytic activity of the enzyme) is used to prepare the tests in this phase of the project; and using the Spectroquant PROVE 100 spectrophotometer, the decrease in the concentration of pNPB is analyzed through the absorbances measured at given times and at the optimal temperatures of the enzymes for the different buffer solutions.

The tests carried out in this project are classified according to the enzyme used (lipase or cutinase) and the buffer solution (Tris-HCl or PBS). For each test, the pH of both dilutions and the pNPB concentration are fixed. The pH level of the test medium has been set at 7.6, since this value, within the pH activity range was found to offer good enzymatic activity in both enzymes. The value of the pNPB concentration is based on the scientific article "Functionalization of mesoporous silica for lipase immobilization. Characterization of the support and catalysts", which uses a concentration of 0.4 mM of paranitrophenol substrate to define esterase activity [14]. The variable values are the amount of enzyme added to the assays and the temperatures that will allow obtaining the optimal values of both enzymes (37 & 55 ºC). Finally, the total test volume size has been set at 6 mL, allowing the collection of enough 0.25 mL samples along the test period, as well as additional samples if required. Considering this amount of sample, a 200 mL solution containing 0.4 mM pNPB was prepared. According to the number of units of enzymatic activity estimated to degrade all the moles of the substrate (being 0.04 U in both enzymes), and the remainder is the assay buffer medium. The calculations to define the assays are shown below.

Lipase B

$$\frac{0{,}04\ U}{9\ \frac{U}{mg}} = 0{,}00\widehat{4}\ mg = 4{,}4\ \mu g \quad (1)$$

Where 9 U/mg is the enzyme concentration used for this set of experiments.

An analytical balance is used to weigh an amount of enzyme of approximately 1000 µg, which is dissolved in 4 mL of PBS medium, obtaining a concentration of 0.25 µg/µL.

$$\frac{4{,}4\ \mu g}{0{,}25\ \mu g/\ \mu L} = 17{,}6\ \mu L \quad (2)$$

The buffer medium to add for each enzyme concentration is:

$$(Simple\ enzyme\ concentration)\ 6000 - 200 - 17{,}6 = 5782{,}4\ \mu L \quad (3)$$

$$(Double\ enzyme\ concentration)\ 6000 - 200 - 35{,}2 = 5764{,}2\ \mu L \quad (4)$$

Cutinase 2P

$$\frac{0{,}04\ U}{20\ \frac{U}{mg}} = 0{,}002mg = 2\ \mu g \quad (5)$$

Where 20 U/mg is the enzyme concentration used for this set of experiments

An analytical balance is used to weigh an amount of enzyme of approximately 1000 µg, which is dissolved in 4 mL of PBS medium, obtaining a concentration of 0.25 µg/µL.

$$\frac{2\ \mu g}{0{,}25\ \mu g/\ \mu L} = 8\ \mu L \quad (6)$$

The buffer medium to add for each enzyme concentration is:

$$(Simple\ enzyme\ concentration)\ 6000 - 200 - 8 = 5792\ \mu L \quad (7)$$

$$(Double\ enzyme\ concentration)\ 6000 - 200 - 16 = 5784\ \mu L \quad (8)$$

The test duration is set at 60 min, and the compound absorbance is measured over time by taking 9 samples + 1 prior sample before adding the enzyme (0, 2.5, 5, 7.5, 10, 12.5, 15, 20, 30, 60 min). The assay times are designed to account for the enzyme saturation effect and the decrease in available substrate, prioritizing sample collection within the first 15 minutes of the assay. This stabilizing effect on enzyme activity has been demonstrated throughout the project. The stirring speed is 200 rpm. To stop reaction at desired time, it is necessary to denature the enzyme by changing the pH of the buffer dilutions. For this purpose, the compounds HCl and NaOH could be used, which acidify or alkaline the dilution medium. For the project, NaOH 1 M is used, as it provides better coloration in the samples, facilitating the spectrophotometric technique to measure absorbance. Each sample withdraws 250 µL, which is then added to 750 µL of enzyme denaturing agent, this is the ideal proportion to obtain the denaturing effect.

To analyze the decrease in pNPB concentration in the Tris and PBS assays, for this purpose, a calibration curve is used where, once the absorbances at different concentrations are known, a linear line is obtained according to the Lambert-Beer law, therefore two standards were designed for each buffer solution. The substrate concentrations in the standards cover the entire range of concentrations required to study the drop in pNPB concentration after enzyme application. The chosen range extends from 0 mM to 0.5 mM pNPB, considering that the initial assay concentration is set at 0.4 mM. As an example, the standard curve used to analyze the absorbance-time data for the Tris-HCl buffer solution is shown. The graph shows the absorbances obtained for the Tris-HCl+pNPB buffer at 405 nm (blue) and Tris-HCl+pNPB (25%) + NaOH (75%) (grey).

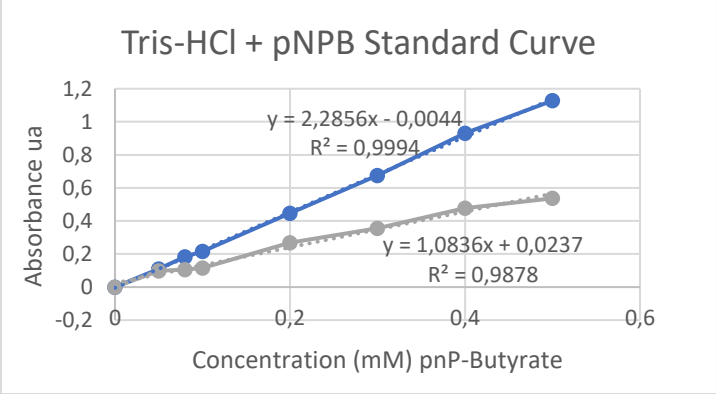


Figure 3: Concentration-time graph of the lipase B assay in Tris-HCl buffer

To obtain the test concentrations from the sampling points, the calibration line of Tris-HCl+pNPB (25%) + NaOH (75%) is used because the samples to be studied are in that proportion for the denaturation of the enzyme to occur. The following linearized equation of the Lambert-Beer Law is utilized to obtain concentration from the absorbance:

$$A = m * C + b \tag{9}$$

Once the concentrations are obtained, the equations of the Michaelis-Menten model are used to find the key parameters that define the enzymes (maximum speed and the Michaellis constant) that relate the reaction rate with the substrate concentration as seen in the following equation:

$$v = \frac{v_{max} * [S]}{K_m + [S]} \tag{10}$$

In order to estimate rates, experimental data obtained in the Concentration-Time graph were fit considering polynomial expressions. By means of simple derivation reaction rate v, related to the substrate concentration, was calculated in the following way:

$$v = \frac{-d[S]}{dt} \tag{11}$$

To estimate the values of K_m and v_{max}, least squares adjustments based on linearizations (the equation is transformed by rearranging its terms to represent a linear graph) of the Michaelis-Menten equation can be used. In this project, only the linearization by the Lineweaver-Burk method of the Michaelis-Menten equation has been utilized, for this purpose the double inverse formula will be applied:

$$\frac{1}{v} = \frac{K_m}{v_{max}} + \frac{[S]}{v_{max}} \tag{12}$$

Once the enzyme kinetic parameters have been obtained, the same procedure is applied to all the tests carried out in this project, considering the standard curve of each dissolution buffer and the absorbance points obtained during sample collection.

4.3. Development of Microplastics tests

Once the enzymatic activity parameters have been obtained, the appropriate medium for performing the PET assays and the optimal enzyme temperatures will be selected. Synthetic PET samples both in water and Tris-HCl buffer reservoirs are prepared and analyzed (after phase 2, it was analytically confirmed that this buffer is the most suitable for achieving greater enzyme efficacy). Once the synthetic samples for use in the MP degradation tests are ready, the particle concentrations in both artificially prepared reservoirs are calculated. This allows the amount of MP required in each experiment to be determined and controlled, making the necessary dilutions.

To determine the concentration of MP, the Olympus DSX1000 microscope is used, which allows the analysis of the shape of the particles that can be found (MP, salts, filter remains...) as

well as observing the quantity of elements in the analyzed quadrant and the microscope by infrared spectroscopy by the Fourier transform (FTIR), allows to ensure and discriminate that the elements found were PET remains, ruling out salts or cellulose remains. It was observed that the synthetic reservoirs had a high concentration of MP, making it necessary to use one of the subsampling strategies analyzed in the document "Assessment of Subsampling Strategies in Microspectroscopy of Environmental Microplastic Samples" [15]. The subsampling strategy of analyzing 25% of the filter was chosen. To ensure a correct comparison, it will be applied to all samples to be analyzed. The concentrations obtained from the synthetic reservoirs are shown in the following graph, presenting concentrations very high compared to those that will be used in the tests.

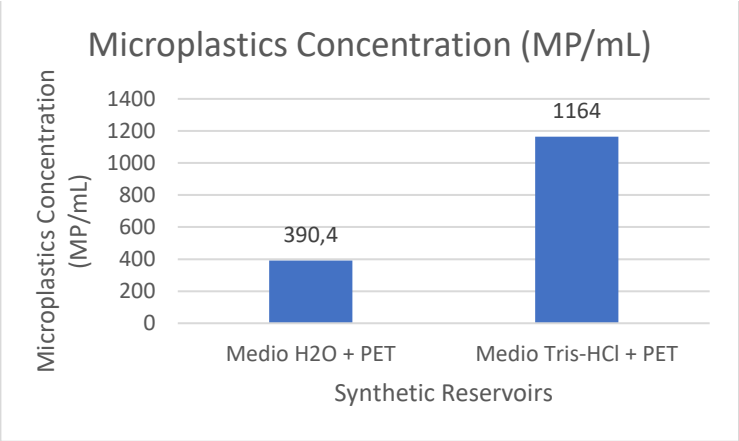


Figure 4: MP concentration in synthetic reservoirs

For the tests conducted in this project, two synthetic PET reservoirs were prepared considering a lower PET concentration (0,453 MP/mL) and a higher one (4,53 MP/mL) according to the calculations explained below. The procedures for reducing the concentration of MPs in the synthetic reservoirs previously analyzed (390.4 MPs/mL in the H_2O + PET medium and 1164 MPs/mL in the Tris-HCl + PET buffer) are presented below, thus obtaining the initial theoretical concentrations for the tests. To determine these PET quantities, the number of filter squares planned to be analyzed was counted to determine the high and low PET concentrations indicated in the previous paragraph.

H_2O + PET medium

The filter grid squares analyzed are 132. This number of squares analyzed is what allows the theoretical fixed concentrations of high and low MP concentration per square indicated previously to be set:

- Low concentration of PET. Final concentration (0,44 MP/mL)

$$390{,}4\ \frac{MP}{mL} * V = 300mL * 0{,}44\ \frac{MP}{mL} \quad (13)$$

V= 0,338 mL

- High concentration of PET. Final concentration (4,4 MP/mL)

$$390{,}4\ \frac{MP}{mL} * V = 300mL * 0{,}44\ \frac{MP}{mL} \quad (14)$$

V=3,38 mL

Tris-HCl + PET buffer

The squares of the filter grid that are analyzed are 136. This number of squares that are analyzed are those that allow the fixed concentrations of high and low concentration of MP per square indicated previously to be set:

- Low concentration of PET. Final concentration (0,453 MP/mL)

$$1164\frac{MP}{mL} * V = 300mL * 0{,}453\ \frac{MP}{mL} \quad (15)$$

V= 0,116 mL

- High concentration of PET. Final concentration (4,53 MP/mL)

$$1164\ \frac{MP}{mL} * V = 300mL * 4{,}53\ \frac{MP}{mL} \quad (16)$$

V=1,16 mL

Once the dilutions calculated previously have been prepared, the synthetic reservoirs will be ready. For the PET degradation tests, the total volume was set at 6 mL, considering both 0,6 mL of enzyme solution (at a concentration of 0.25 µg/µL), and the remaining volume corresponding to the medium containing the PET to be tested. The test temperatures are the optimal temperatures for enzymes, and, as in the tests to obtain the enzyme kinetic parameters, the stirring speed is 200 rpm. Under the test conditions, the enzymatic activity of lipase B and cutinase 2P is studied at temperatures of 55 ºC and 37 ºC. These temperatures, as indicated in previous sections, are the optimal operating temperatures for these enzymes, respectively. The tests to be performed to analyze how the enzyme affects PET degradation are now defined.

Negative Controls:

- 2 assays with only distilled water in Falcon tubes, at temperatures of 55°C and 37°C, respectively.
- 2 assays with H_2O + PET medium (low concentration) without enzyme at conditions of 55°C and 37°C.
- 2 assays with H_2O + PET medium (high concentration) without enzyme at conditions of 55°C and 37°C.
- 2 assays with only Tris-HCl medium in Falcon tubes, at temperatures of 55°C and 37°C.
- 2 assays with Tris-HCl + PET medium (low concentration) without enzyme at conditions of 55°C and 37°C.
- 2 assays with Tris-HCl + PET medium (high concentration) without enzyme at conditions of 55°C and 37°C.

MP assays in Tris-HCl medium:

- Assays at 37°C:
  - 2 assays with the enzyme lipase B with high and low PET concentrations.
  - 2 assays with the enzyme cutinase 2P with high and low PET concentrations.
- Assays at 55°C:
  - 2 assays with the enzyme lipase B with high and low PET concentrations.
  - 2 assays with the enzyme cutinase 2P with high and low PET concentrations.

Once the quantities and number of tests to be performed in this phase of the project have been defined, the test time is set at 48 hours. Once this time has elapsed, the vacuum filtration technique is applied to each collected sample, and subsequently each filter is dried in oven for 24-48 hours. After this time, filters are removed from the oven and placed in a desiccator within *Petri* dishes to reach room temperature and prevent moisture from being absorbed from the medium. Sample analysis will be analyzed using Olympus DSX1000 microscopes and a Jasco infrared spectrophotometer. As was done for the analysis of the synthetic reservoirs, a subsampling strategy was used to analyze 25% of the filter, since the PET concentration in the tests was high.

5. Results

This section presents the results of the tests and graphs performed throughout the project. For a correct understanding of the results and to facilitate an appropriate reading order, this chapter has been divided into three main parts, taking into account the chronological order in which the results were obtained: 1) the results of the enzymatic kinetic parameters of the enzymes tested; 2) the percentages and sizes of PET found in the enzymatic degradation tests; 3) the tables with the project budgets.

5.1 Enzyme kinetics sample results

This section shows the results of the enzyme kinetics for the tests with a single and double amount of enzyme.

Table 2: Parameters of enzyme kinetics of single-amount enzyme assays

Single-amount enzyme assays								
	Tris-HCl buffer				**PBS buffer**			
	Lipase B		Cutinase 2P		Lipase B		Cutinase 2P	
Temperature	v max $(\frac{\mathrm{mM}}{min})$	Km (mM)	v max $(\frac{\mathrm{mM}}{min})$	Km (mM)	v max $(\frac{\mathrm{mM}}{min})$	Km (mM)	v max $(\frac{\mathrm{mM}}{min})$	Km (mM)
55 ºC	0,0120	0,0920	$4{,}916*10^{-3}$	0,2250	$5{,}000*10^{-3}$	0,0550	$9{,}020*10^{-4}$	0,0125
37 ºC	0,0030	0,2120	$6{,}420*10^{-4}$	$1{,}475*10^{-3}$	-	-	$3{,}621*10^{-4}$	0,0124

Table 3: Parameters of enzyme kinetics of double-amount enzyme assays

Double-amount enzyme assays								
	Tris-HCl buffer				**PBS buffer**			
	Lipase B		Cutinase 2P		Lipase B		Cutinase 2P	
Temperature	v max $(\frac{\mathrm{mM}}{min})$	Km (mM)	v max $(\frac{\mathrm{mM}}{min})$	Km (mM)	v max $(\frac{\mathrm{mM}}{min})$	Km (mM)	v max $(\frac{\mathrm{mM}}{min})$	Km (mM)
55 ºC	0,4060	1,3150	$6{,}375*10^{-3}$	0,2350	$3{,}700*10^{-3}$	0,6880	$4{,}730*10^{-4}$	0,1150
37 ºC	0,0010	0,0920	$1{,}518*10^{-3}$	0,0469	-	-	$5{,}163*10^{-4}$	0,0533

5.2 PET sample results

The following tables compile all the results showing the % of both cellulose and PET particles found in the samples.

Table 4: Percentage of particles in high concentration PET tests

High concentration PET	H_2O + PET 55 ºC	H_2O + PET 37 ºC	Tris-HCl + PET 55 ºC	Tris-HCl + PET 37 ºC
% Cellulose in tests	46,42%	51,00%	46,15%	48,71%
% PET in test	53,58%	49,00%	53,85%	51,29%
High concentration PET	Cutinase 2P 55 ºC	Cutinase 2P 37 ºC	Lipase CalB 55 ºC	Lipase CalB 37 ºC
% Cellulose in tests	40,00%	44,00%	40,00%	46,80%
% PET in test	60,00%	56,00%	60,00%	53,20%

Table 5: Percentage of particles in low concentration PET tests

Low concentration PET	H_2O + PET 55 ºC	H_2O + PET 37 ºC	Tris-HCl + PET 55 ºC	Tris-HCl + PET 37 ºC
% Cellulose in tests	57,89%	56,62%	61,70%	67,34%
% PET in test	42,11%	43,38%	38,30%	32,66%
Low concentration PET	Cutinase 2P 55 ºC	Cutinase 2P 37 ºC	Lipase CalB 55 ºC	Lipase CalB 37 ºC
% Cellulose in tests	50,00%	48,25%	45,23%	55,88%
% PET in test	50,00%	51,75%	54,77%	44,12%

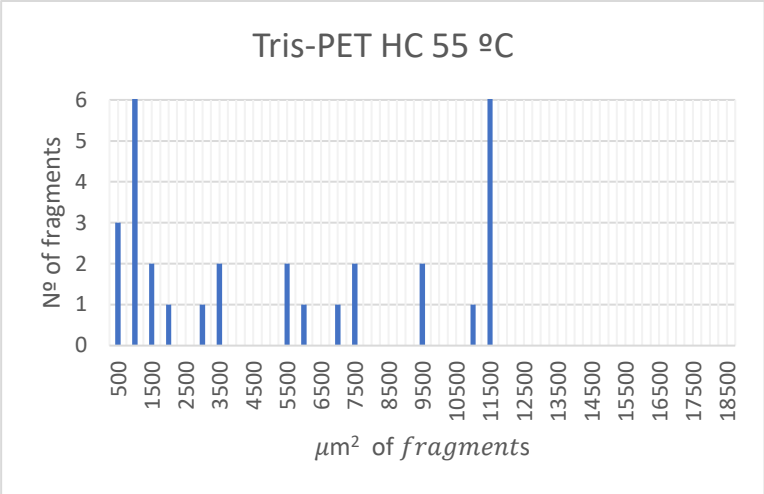
Table 6: Summary of PET concentration results in Lipase B laboratory tests

Temperature	Defined PET concentration	Concentration Tris+PET WITHOUT enzyme (MP/mL)	Concentration lipase test (MP/mL)	Concentration variation lipase B test (MP/mL)
37 ºC	Low concentration PET	11,975	14,413	2,437
	High concentration PET	13,335	23,408	10,072
55 ºC	Low concentration PET	12,767	13,145	0,378
	High concentration PET	18,309	20,400	2,091

Table 7: Summary of PET concentration results in Cutinase 2P laboratory assays

Temperature	Defined PET concentration	Concentration Tris+PET WITHOUT enzyme (MP/mL)	Concentration cutinase test (MP/mL)	Concentration variation lipase B test (MP/mL)
37 ºC	Low concentration PET	11,975	12,420	0,445
	High concentration PET	13,335	24,640	11,305
55 ºC	Low concentration PET	12,767	8,333	-4,433
	High concentration PET	18,309	19,600	1,291

Below are the frequency tables for the particle sizes analyzed in the filters. The size of the fluorine resin and cellulose fibers have been ignored, only PET fragments have been measured. This study of fragment morphology will determine whether the previously obtained increase in PM concentration is consistent with a decrease in PM size.



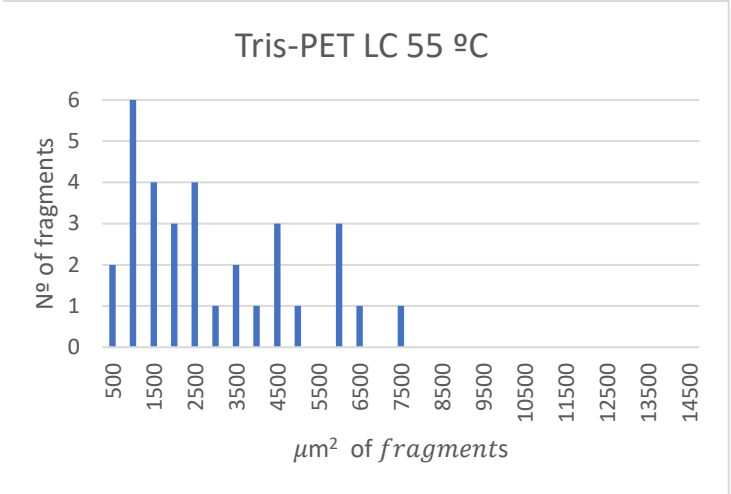
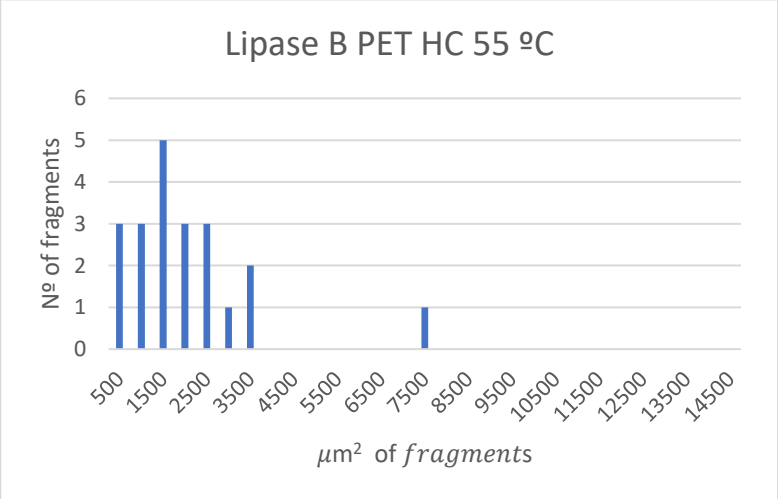


Figure 5: Size of MP fragments found in Tris-HCl buffer at 55 ºC

423



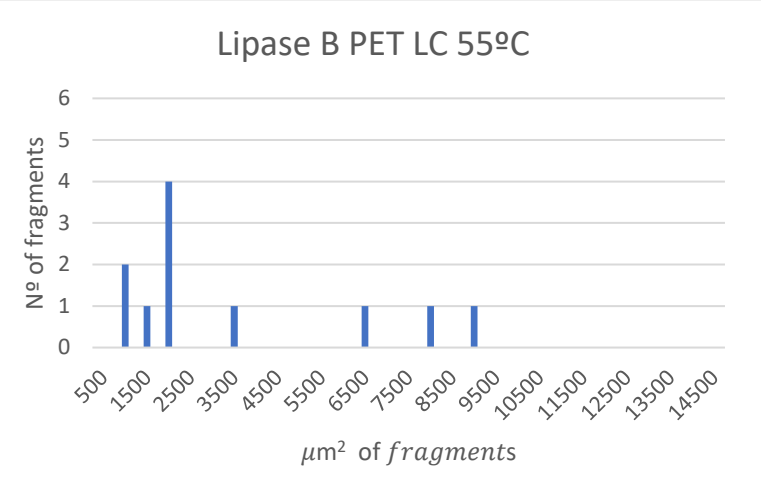
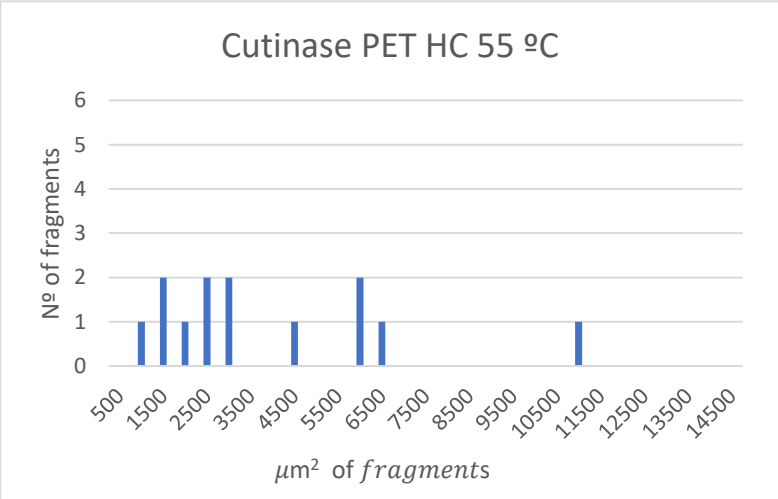


Figure 6: Size of MP fragments found in lipase B assays at 55 ºC

424



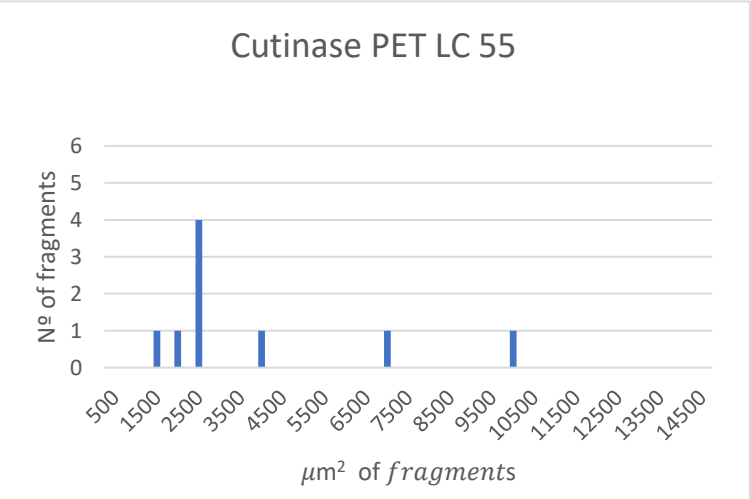
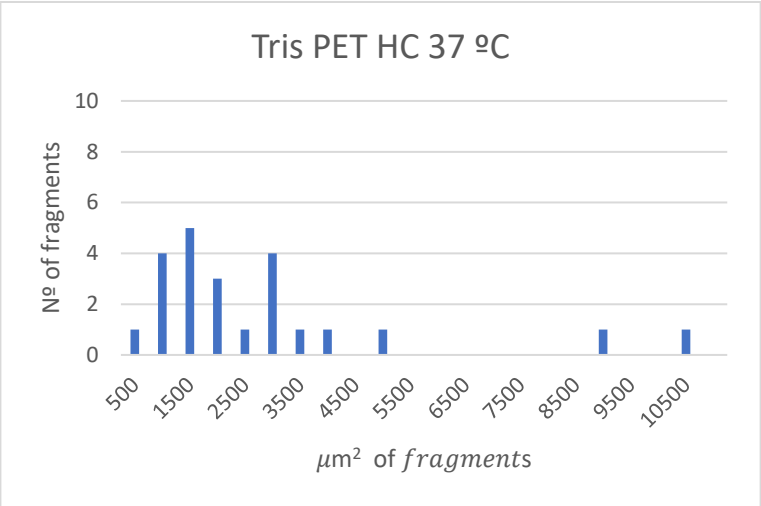


Figure 7: Size of MP fragments found in cutinase 2P assays at 55 ºC

425



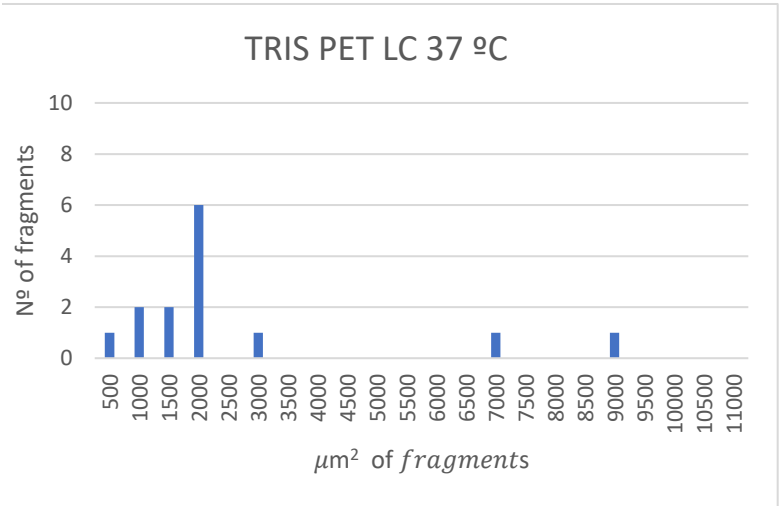
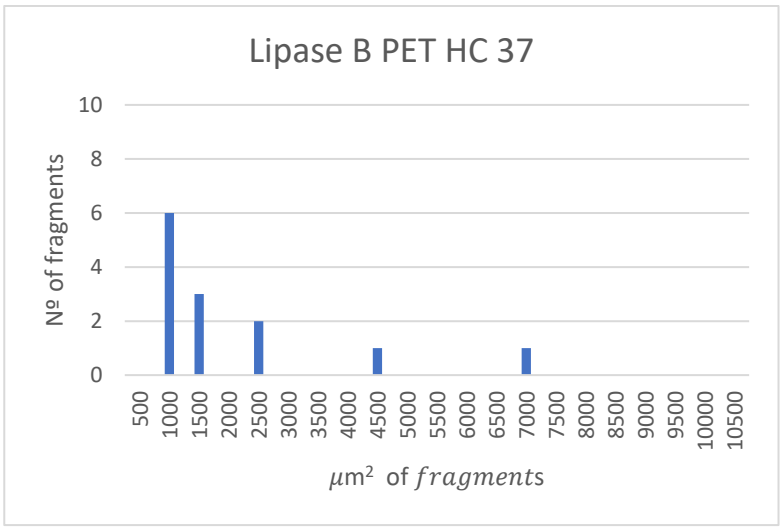


Figure 8: Size of MP fragments found in Tris-HCl buffer assays at 37 ºC



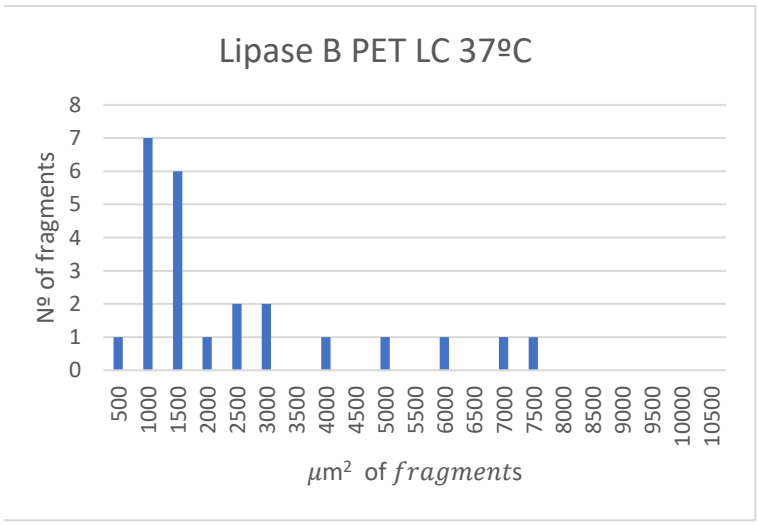
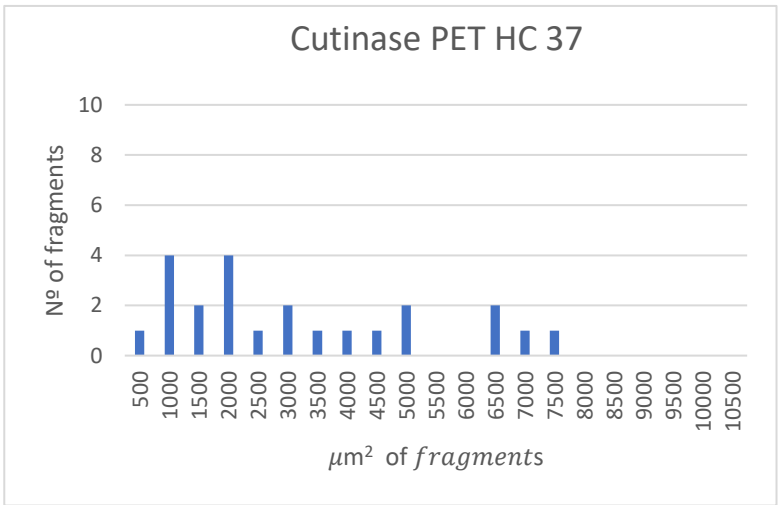


Figure 9: Size of MP fragments found in lipase B assays at 37 ºC



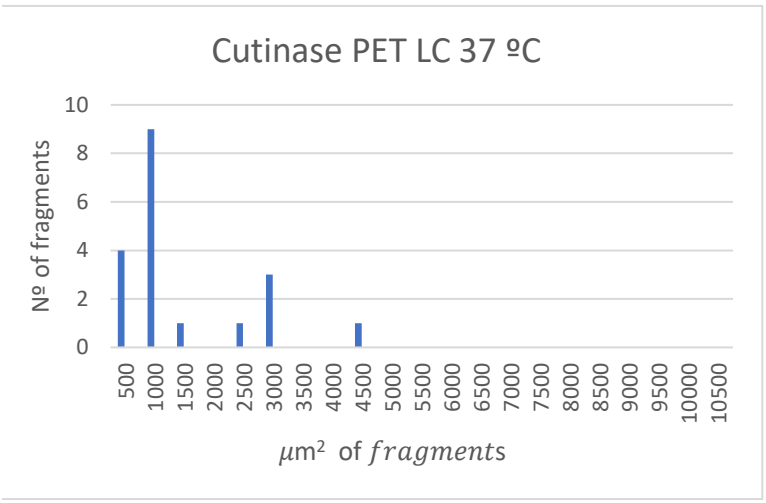


Figure 10: Size of MP fragments found in cutinase 2P assays at 37°C

5.3 Economics results

This section shows the costs required to perform all the tests relevant to the enzymatic degradation of MPs on a laboratory scale, the cost of the devices used, as well as the costs of the tools required for the evolutionary measurement of degradation.

Table 8: Budget for devices used

Name	Individual Price €	Units	Total Price €
OLYMPUS DSX 1000 microscope	60.000,00	1	60.000,00
JASCO infrared spectrophotometer (FTIR + microscope)	101.200,75	1	101.200,75
Spectroquant PROVE 100 Spectrophotometer	6.944,16	1	6.944,16
Mettler Toledo XS205 analytical balance	10.354,40	1	10.354,40
MERCK Direct-Q Water Purifier	13.860,00	1	13.860,00
Ectractio hood	5.447,95	1	5.447,95
Laboratory Incubator	1.399,00	1	1.399,00
SKI 4 Orbital Incubator	4.354,79	1	4.354,79
GLP 21 pH Meter	1.125,00	1	1.125,00
			204.686,05

Table 9: Budget for laboratory tools, compounds and enzymes

Name	Individual Price €	Units	Total Price €
Laboratory mortar	697,00	1	697,00
Petri dishes	2,55	44	112,20
Sterile 15 ml *FALCON* tubes	0,29	118	34,22
Eppendorf Protein LoBind tubes	0,12	160	19,84
Liquid nitrogen (25 L tank fill)	310,33	1	310,33
Tris base (100 g)	42,00	1	42,00
Sodium phosphate monobasic (500 g)	78,40	1	78,40
Sodium hydroxide (NaOH) (500 g)	83,80	1	83,80
Hydrochloric acid (HCl)-37% (500 mL)	640,00	1	640,00
Candida antarctica lipase B, recombinant from Aspergillus oryzae (50 mg)	229,00	1	229,00
Cutinase 2P (10,000 U)	59,00	1	59,00
4-Nitrophenyl butyrate (1 g)	120,00	1	120,00
			2.425,79

6. Discussion

Analysis of enzyme kinetics sample results

Results show that the maximum rates of both enzymes are higher in the assays using Tris-HCl buffer than those obtained with PBS,. Consequently, to obtain the best enzymatic activity of the enzymes in PET degradation assays, the Tris-HCl buffer was used exclusively in assays using PET as the substrate.

Furthermore, as the amount of enzyme increased, the reaction rate also increased in assays where each enzyme was at its optimal temperature, allowing for an improvement in the efficiency of enzymatic degradation to be considered in assays using PET substrates.

Analysis of PET sample results

The results obtained from the previous tests confirm that the Tris-HCl medium is suitable for the proper functioning of the enzymes in their enzymatic activity. Results show an increase in the concentration of microplastics in the tests where each enzyme was applied separately (except in the case of cutinase) at a temperature of 55 ºC. From this it can be concluded that the cutinase enzyme is not acting at its optimal temperature, which is 37 ºC, and its enzymatic activity may have been reduced.

At 55°C, lipase B assays do show an increase in both microplastics and a reduction in their size. Microplastic sizes approach 1500 μm^2 with the enzyme, while without the addition of enzyme, they reached sizes of 11,500 μm^2.

For experiments using cutinase at 55°C, particle sizes do not experience a reduction in size compared to lipase B, where a reduction is observed. This is consistent with the previously obtained result of the microplastic quantity, since the optimal temperature for cutinases is 37°C, meaning that excessively high temperatures (55°C in these assays) can denature the enzyme, limiting its activity.

Results at 37°C show an increase in the number of PET particles, as well as a reduction in their size. This is the same for both enzymes, indicating that the operating temperature range of lipase B is broader than that of the cutinase enzyme. Meanwhile, in the optimal temperature tests for cutinase, at its ideal temperature, the particle size decreased compared to the results at 55°C.

7. Conclusions

In this section, the conclusions obtained in the enzymatic degradation tests of PET will be presented:

- After performing enzyme activity tests in Tris-HCl and PBS solutions, the maximum rates of enzymatic reactions in the PBS buffer solution were lower than those obtained in the Tris-HCl buffer (in some tests, no effect was even detected). This is mainly due to the composition of both solutions, and the salts in the PBS medium can affect enzyme activity. It was confirmed that the Tris-HCl medium is suitable for the proper functioning of the enzymes. Sodium and potassium cations could act as cofactors for the enzymes affecting chemical rates.
- The concentrations of microplastics in the tests increased compared to the negative control blanks. This means that, under the appropriate temperature conditions, the hydrolytic enzymes exhibit high enzymatic activity, degrading and breaking the primary bonds of PET. This conclusion is also supported by the reduction in particle size shown in the graphs of the frequency tables of the tests. Hence, a left shift in the size distribution within the corresponding histograms can be seen, showing a reduction in the average size of PET particles. This increase, which at first could be harmful because the concentration of microplastics increases and the size decreases and could affect the bloodstream, serves to facilitate other living beings to access the secondary bonds and the degradation of PET can be complete, thereby obtaining the components terephthalic acid and ethylene glycol.
- No traces of polypropylene were found in the tests carried out in this project, which could indicate that the tubes where the enzymatic reactions are carried out do not

suffer degradation at the optimal temperatures of the enzymes at 37 and 55 degrees Celsius (mainly traces of PET and cellulose were found in the analyses of these). According to this, a certain selectivity towards the type of bonds present in PET can be inferred.

- Regarding subsampling strategies for the analysis of test filters, it is recommended to increase the concentration of MP to be analyzed so that only 25% of the sample needs to be analyzed and the margin of error presented is not high, without having to analyze up to 50%.
- The use of purified enzymes instead of living organisms has allowed for good control of the assay parameters, allowing for the application of optimal enzyme conditions instead of those of the organism.
- From an economic perspective, this work indicates that approximately €207,111.84 was spent on the completion of this project, mainly on the devices used throughout.

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