Determination of key-thermodynamic parameters using a kinetic modeling approach to describe the post-consumer poly(ethylene terephthalate) hydrolysis catalyzed by cutinase from *Humicola insolens*

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ABSTRACT

The search for a straightforward technology for post-consumer poly(ethylene terephthalate) (PC-PET) degradation is essential to develop a circular economy. In this context, PET hydrolases such as cutinases can be used as bioplatforms for this purpose. *Humicola insolens* cutinase (HiC) is a promising biocatalyst for PC-PET hydrolysis. Therefore, this work evaluated a kinetic model, and it was observed that the HiC seems not to be inhibited by any of the main PET hydrolysis products such as terephthalic acid (TPA), mono-(2-hydroxyethyl) terephthalate (MHET), and bis-(2-hydroxyethyl) terephthalate (BHET). The excellent fitting of the experimental data to a kinetic model based on enzyme-limiting conditions validates its employment for describing the enzymatic PC-PET hydrolysis using two-particle size ranges (0.075-0.250, and 0.250-0.600 mm) and temperatures (40, 50, 55, 60, 70, and 80 ºC). The Arrhenius law provided a reliable parameter (activation energy of 98.9 ± 2.6 kJ mol⁻¹) for enzymatic hydrolysis, which compares well with reported values for chemical PET hydrolysis. The thermodynamic parameters of PC-PET hydrolysis corresponded to activation enthalpy of 96.1 ± 3.6 kJ mol⁻¹ and activation entropy of 10.8 ± 9.8 J mol⁻¹ K⁻¹. Thus, the observed rate enhancement with temperature was attributed to the enthalpic contribution, and this understanding is helpful to the comprehension of enzymatic behavior on hydrolysis reaction.

*Keywords:* PET biodegradation; *Humicola insolens* cutinase; Kinetic parameters; Heterogeneous biocatalysis.
1. Introduction

Poly(ethylene terephthalate) (PET) is one of the significant plastics produced worldwide, and it is largely used for the packaging market and the textile industry. The annual global PET production is almost 70 million tons [1], and it is expected to increase over the years due to its great packaging demand [2]. As a consequence of this, an enormous amount of post-consumer PET (PC-PET) is discarded in inappropriate places or buried in landfills, and the minimization of such wastes have been focused countless efforts in order to implement a circular economy [3–6]. Some reports claim that bioremediation can help mitigate marine and terrestrial contamination [7, 8]. In this context, enzymatic catalysis, specifically the biodepolymerization process, dawns as one of the most promising technologies in a plastic circular economy. In the last decade, the use of esterases (EC 3.1.1.1) [9–11], lipases (EC 3.1.1.3) [12–18] and cutinases (EC 3.1.1.74) [14–21] as biocatalysts have been extensively investigated in an alternative eco-friendly route, the PET hydrolysis. Currently, cutinases from the thermophilic actinomycete Thermobifida fusca [22], from leaf-branch compost [1], from the thermophilic fungus Humicola insolens [21], and the mesophilic IsPETase cloned from Ideonella sakaiense [1, 23] show acceptable efficiency to degrade PET waste, such as packaging materials.

PET enzymatic hydrolysis is described as a heterogeneous reaction system in which the biocatalyst in an aqueous environment catalyzes with the insoluble and macroscopic polyester, containing highly ordered (crystalline) and less ordered (amorphous) regions [24], being the last ones more accessible. Therefore, PET erosion probably occurs similarly to cellulose degradation [24]. This phenomenon consists of chain disruption and loosening, making the individual substrate molecule more accessible and available for interactions with degrading enzymes [25]. Moreover, enzymatic hydrolysis of PET occurs with the generation of new free hydroxyl and carboxyl groups at the polymer surface through the cleavage of ester backbones, which results in increased PET hydrophilicity [26]. The resulting low molecular weight products of the enzymatic hydrolysis comprise ethylene glycol (EG), and terephthalic acid (TPA) that are the PET synthesis monomers, and beyond these, mono(2-hydroxyethyl) terephthalate (MHET), and bis(2-hydroxyethyl) terephthalate (BHET) that are the main intermediate reaction products.

The depolymerization of water-insoluble substrates using enzymes, such as cellulases, chitinases, xylanases, and depolymerases, occurs via two-steps: adsorption and hydrolysis. In
the first moment occurs the adsorption of the enzyme on the hydrophobic surface of the substrate. The second step is the hydrolysis of the polymer chain at the active site of the enzyme [27]. Therefore, the hydrophobicity of the enzyme surface influences the interaction with hydrophobic polyesters [28]. Thus, enzymatic hydrolysis is a route for recycling PET waste that depends strongly on the substrate surface availability [29, 30].

The mobility of PET chains is also a relevant factor for the biodegradability of polyesters [31]. The chain mobility of the polymer is determined by the crystallinity of the polymer, and it increases at temperatures close to its glass transition temperature ($T_g$) [26, 32, 33]. The increase of the reaction temperature of PET hydrolysis increases polymer chain flexibility, which favors the diffusion of water molecules between the polymer chains through the weakening of the hydrogen bonds, as well as randomizing and increasing the flexibility of the PET chain, thus, enhancing the enzyme accessibility to the ester bonds [26].

In light of the challenges mentioned above to describe PC-PET enzymatic hydrolysis, this study initially evaluated the stirring speed effect and the availability of the substrate concentration on the reaction rate. Thus, a kinetic analysis was performed through a model based on enzyme-limiting conditions to determine key-kinetic parameters, which are relevant for developing an industrial process of PET depolymerization. The product inhibition was also evaluated. Although some studies have suggested a mathematical approach to describe PET hydrolysis, to the best of our knowledge, none of them determined the activation parameters for this reaction. Therefore, the investigation of *Humicola insolens* cutinase (HiC) ability to undergo efficient catalysis is reported here over the temperature range of 40 to 80 °C. The aim of this study is not only to determine the kinetics of the enzymatic reaction but also to provide critical parameters that must be considered in the design of a scalable process.

2. Materials and methods

2.1. Materials

Terephthalic acid (TPA, purity 98%) and bis-(2-hydroxyethyl) terephthalate (BHET, purity 99.8%) were purchased from Sigma-Aldrich. Mono-(2-hydroxyethyl) terephthalate (MHET) was synthesized by our group through the enzymatic hydrolysis of BHET. The
commercial liquid preparation with *Humicola insolens* cutinase (product Novozym® 51032) was kindly provided by Novozymes (Araucária, Brazil).

The post-consumer PET (PC-PET) was originated in an industrial mechanical recycling plant and was composed of different (i.e., different thicknesses, colors, etc.) packages. The PC-PET presented high crystallinity (41.1%) and high molar mass (43,379 g mol\(^{-1}\)), as described by Castro and collaborators [18]. The material was received as flakes, grinded in a knife mill, and sieved into two fractions with different granulometric size ranges: F1: 0.075-0.250 mm, and F2: 0.250-0.600 mm.

2.2. *Quantification of the enzymatic hydrolysis products*

The quantification of TPA, MHET, and BHET concentrations was carried out in a Waters High-Performance Liquid Chromatograph equipped with a binary pump (1525 model), and a UV/Visible detector (2489 model) at 254 nm wavelength. The injection volume employed was 20 μL, and the column, an Eclipse Plus C18 column (Agilent Technologies), of 4.6 mm x 250 mm and 5 μm particle diameter, was kept at 30 °C. The analysis methodology was performed using a gradient elution method, and the mobile phases used were acetonitrile and an aqueous solution of formic acid (0.05 vol.%) under a total flow rate of 0.500 mL min\(^{-1}\). The TPA, MHET, and BHET concentrations were determined based on standard curves.

2.3. *Enzymatic characterization*

The protein concentration of HiC commercial liquid preparation was determined according to the Bradford method [34], and the obtained result was 16.8 mg mL\(^{-1}\). The polyacrylamide gel electrophoresis analysis in the presence of sodium dodecyl sulphate (SDS-PAGE) indicated that the predominant protein of HiC preparation has a molar mass of around 18 kDa.

The hydrolytic activity of HiC was assessed as the BHET hydrolysis capacity, releasing MHET and ethylene glycol, in 200 mmol L\(^{-1}\) phosphate buffer, pH 7.0. The reaction was carried out in a 50 mL glass reaction vessel with mechanic stirring (EasyMax™ 102, Mettler Toledo) with an initial BHET concentration of 8 mmol L\(^{-1}\) and enzyme concentration of 0.05 g\(_{\text{protein}}\)
The reaction was monitored by the quantification of TPA, MHET, and BHET by HPLC, according to the methodology described in item 2.2. One unit of activity was defined as the amount (μmol) of MHET formed per minute, and the result obtained for HiC specific activity was 1.9 ± 0.2 U mg\textsubscript{protein}\textsuperscript{-1}. The enzyme activity remained constant during all the experiments.

2.4. \textit{PET hydrolysis}

The PC-PET hydrolysis was carried out in a 10 mL reactor provided with magnetic stirring (EasyMax\textsuperscript{TM} 102, Mettler Toledo), employing HiC as a biocatalyst diluted in phosphate buffer solution. All tests were carried out using 200 mmol L\textsuperscript{-1} phosphate buffer (pH 7.0), and HiC concentration of 1.0 mg\textsubscript{protein} mL\textsuperscript{-1}. At appropriate intervals, samples of the reaction medium were withdrawn and diluted in methanol before chromatographic analysis. The sum of the TPA, MHET, and BHET concentrations was used to determine the initial reaction rates.

The TPA yield was defined as described in Equation 1, where [TPA] and [PET]\textsubscript{0} refer to the concentration of TPA and the initial concentration of PET on mass basis (g L\textsuperscript{-1}), respectively. \(M_{TPA}\) and \(M_{PET}\) are the molar mass of TPA (166.1 g mol\textsuperscript{-1}) and the PET repeat unit (192.2 g mol\textsuperscript{-1}), respectively [35].

\[
TPA \text{ Yield(\%)} = \frac{[TPA]_{\text{mol}}}{M_{TPA}} \times 100
\]

2.4.1. Effect of substrate concentration on PET hydrolysis

The effect of PET concentration on the enzymatic hydrolysis was evaluated using different PET concentrations: 10, 35, 50, 80, 100, 120, 150, 200, 220, 250 g L\textsuperscript{-1}. For all assays, the reactor stirring speed was 800 rpm, and the reaction temperature was kept at 70 °C.

2.4.2. Effect of stirring speed

The diffusion effect was studied under three different stirring speeds: 100, 400, and 800 rpm, and various substrate concentrations (10, 35, 50, 80, 100, 120, 150, 200, 220, 250 g L\textsuperscript{-1}), at 70° C.
2.4.3. Kinetic study of enzymatic PET hydrolysis and product inhibition

Barth and collaborators [30] proposed a model for the heterogeneous enzymatic PET hydrolysis. The following assumptions were made in order to simplify the mathematical representation [36]:

- Considering that the catalytic site of HiC has the unique function of producing oligomers that can be represented as the product (P), the resulting system could be modeled analyzing only the hydrolysis and adsorption steps with no incorporation of an extra kinetic parameter.

- The substrate concentration is based on the surface concentration accessible to enzymes for adsorption and subsequent catalysis, which considers the effect of substrate particle size.

- The produced oligomers may inhibit the biocatalyst reversibly and competitively, forming an EP complex.

Based on these assumptions, the heterogeneous enzymatic PET hydrolysis can be described by the following reaction scheme:

\[
\begin{align*}
    E + S & \underset{k_d}{\overset{k_a}{\rightleftharpoons}} ES & \quad k & \quad P + E \\
    E + P & \overset{\beta_1}{\underset{\beta_2}{\rightleftharpoons}} PE
\end{align*}
\]  

First, the free enzyme (E) adsorbs to the PET surface. Then, the adsorbed enzyme binds to available ester bonds on the PET surface, forming an enzyme-substrate surface (ES). Finally, the enzyme catalyzes the polyester hydrolysis and releases the product (P). The free enzyme (E) can also bind to the released products becoming briefly unavailable to bind the PET substrate. The forward and reverse reaction rate constants of the formation of the enzyme-substrate complex are \(k_a\) and \(k_d\), respectively. \(k\) is the rate constant of product formation, and \(\beta_1\) and \(\beta_2\) are binding constants for products. According to the proposed reaction scheme, the concentration of the enzyme-substrate complex (ES) can be expressed by Equation 4. Thus, defining the total enzyme concentration (Equation 5) and an adsorption equilibrium constant in terms of the amount of enzyme bound to the substrate (\(K\), Equation 6) or the products (\(\beta\), Equation 7), it was possible to rearrange the equations and define the initial rate as expressed in Equation 8.
\[ [ES] = \frac{K[E_0][S]}{1 + \beta[P] + K[S]} \]  \hspace{1cm} (4) \\
\[ [E_0] = [E] + [ES] + [EP] \]  \hspace{1cm} (5) \\
\[ K = \frac{k_a}{k_d} = \frac{[ES]}{[E][S]} \]  \hspace{1cm} (6) \\
\[ \beta = \frac{\beta_1}{\beta_2} = \frac{[EP]}{[E][P]} \]  \hspace{1cm} (7) \\
\[ v = \frac{d(P)}{dt} = k [ES] = k[E_0] \frac{K[S]}{(1/i) + K[S]} \]  \hspace{1cm} (8)

where \( i \) is the inhibition parameter that can be described for each product formed (P) as shown in Equation 9.

\[ \frac{1}{i} = 1 + \beta \ [P] \]  \hspace{1cm} (9)

If the product did not interact with the enzyme, then \( \beta \) tends to 0; that is, no competitive inhibitory effect is evidenced, and therefore Equation 8 can be rewritten as described in Equation 10.

\[ v = k[E_0] \frac{K[S]}{1 + K[S]} \]  \hspace{1cm} (10)

The kinetic model was adjusted for two different granulometric size ranges in order to test its validity to describe PET hydrolysis. Analysis of specific surface area of selected PET samples was performed in a Mastersizer 2000 analyzer (Malvern Instruments).

The product inhibition was investigated by adding a defined amount of PET hydrolysis products (TPA, MHET, and BHET) to the reaction mixture. After equilibration for 10 min at 70 °C, the HiC was added to the reaction medium. The effect of TPA inhibition was evaluated using TPA concentrations of 10 and 20 mmol L\(^{-1}\). The analysis of MHET as an inhibitor was performed through PET hydrolysis employing MHET concentrations of 5, 10, 15, and 20 mmol L\(^{-1}\). A similar investigation was conducted with BHET concentrations of 2, 10, 20, 30, and 40 mmol L\(^{-1}\). Reactions were carried out at 70 °C with an initial PET concentration of 50 g L\(^{-1}\) with a particle diameter range of 0.075-0.250 mm, and under 800 rpm stirring speed.
2.4.4. **Effect of temperature**

The reactions were carried out at six temperatures (40, 50, 55, 60, 70 and 80 °C), and initial PET concentrations of 10, 35, 50, 80, 100, 120, 150, 200, 220, 250 g L\(^{-1}\). The enzymatic reaction rate \((k)\) was determined as described in Equation 8, at each temperature. Therefore, the temperature dependence of rate constants was calculated through Arrhenius (Equation 11) and Eyring-Polanyi (Equation 12) equations. The Arrhenius constant \((A_0)\) and the activation energy \((E_a)\) were determined using a gas constant \((R)\) of 8.314 J mol\(^{-1}\) K\(^{-1}\). The enthalpy \((\Delta H^\#)\) and entropy \((\Delta S^\#)\) of activation were estimated from derived Eyring-Polanyi equation assuming that the transmission coefficient \((\kappa)\) is equal to 1, for monomolecular reactions [37], and that Boltzmann \((k_B)\) and Planck \((h)\) constants are equal to 1.38.10\(^{-23}\) J K\(^{-1}\) and 6.62.10\(^{-34}\) J s, respectively.

\[
k = A_0 e^{-\frac{E_a}{RT}}
\]

\[
k = \frac{\kappa k_B}{h} T \frac{\Delta S^\#}{R} e^{-\frac{\Delta H^\#}{RT}}
\]

The parameters of the non-linear kinetic model and all the activation parameters were obtained by numerical estimation using the Levenberg-Marquardt algorithm. The data were analyzed by Origin 8.1 software using the convergence criterion of chi-square minimization (tolerance of 10\(^{-9}\)) and the maximum number of iterations equal to 50.

3. **Results and discussion**

3.1. **Effect of PC-PET concentration on enzymatic hydrolysis**

The enzymatic degradation of PET is a heterogeneous catalytic process, in which the reactant is not solvated in the bulk solution. The effectiveness of enzymes is sensitive by continuous enzyme-substrate-product interactions at the PET-water reaction interface, such as enzyme binding, desorption, inhibition, and diffusion [36], and all these phenomena can cause remarkable changes of substrate exposed area. Thus, the effect of substrate concentration (10 – 250 g L\(^{-1}\)) on the concentration of PET hydrolysis products was investigated, and Figure 1 represents the results obtained. The PET hydrolysis products (TPA, MHET, and BHET) concentration had the lowest value at 10 g L\(^{-1}\), while the maximum product concentration
(129.42 mmol L\(^{-1}\)) was obtained with 220 g L\(^{-1}\) of PET after 96h. Moreover, a higher concentration of released products after 96 h was observed than reported for Carniel and collaborators [17] after 14 days of reaction using HiC at 60 °C, and higher than measured for Gamerith and collaborators [38] after 21 days of enzymatic hydrolysis using *Thermobifida cellulosilytica* cutinase at 50 °C, in almost the same enzyme concentration.

**Figure 1**

The results showed that higher substrate concentrations appear to have two positive effects, as expected, (i) faster initial reaction rate and (ii) higher product generation. However, the positive effects of high substrate concentration, concerning the products released, decreased for PET concentrations above 220 g L\(^{-1}\). It seems that interactions between reactants and biocatalysts occur quite differently from their homogenous counterparts, indicating that the diffusion processes in two- or one-dimensional space occur differently [39]. Therefore, the mass transfer limitation was evaluated varying the reaction stirring speed. A more intensive mixing regime should facilitate a better mass transfer inside the reactor, being effective to reduce a potentially high local product concentration surrounding the enzyme active site [36].

### 3.2. **Effect of stirring speed**

The enzymatic PET hydrolysis system is composed of a liquid phase, containing enzymatic extract, and a solid phase consisted of the PET particles. An adequate interaction of the enzyme with the substrate at the solid-liquid interface is essential for efficient enzymatic PET hydrolysis [40]. Therefore, the rate of PET hydrolysis could be affected by diffusion limitations. According to Gan and collaborators [36], the heterogeneous hydrolytic reaction rate is determined by three events in a stirred batch reactor. The first one is the mass enzyme transfer through the stagnant liquid film layer adjacent to the solid substrate, the second event refers to the enzyme adsorption at the substrate surface, and finally, the third one is the catalysis. Thus, with the progress of the enzymatic PET hydrolysis, the amount of available substrate decreases, and the overall reaction rate depends on the enzyme penetration and diffusion inside the solid substrate.
The effect of stirring speed on the PET hydrolysis reaction was investigated with the varying initial concentration of PC-PET (10 to 250 g L\(^{-1}\)) and stirring reaction speed (100, 400, and 800 rpm).

**Figure 2**

According to the results shown in Figure 2, the reactor stirring speeds of 400 and 800 rpm resulted in an efficient interfacial mass transfer with no diffusion restriction for reactions conducted with PC-PET concentrations of up to 150 g L\(^{-1}\). However, when the substrate concentration was higher than 150 g L\(^{-1}\), a large difference between the initial rates obtained under 400 and 800 rpm stirring speed was observed, suggesting a stronger influence of mass transfer contribution compared to the kinetic one. The increase of the initial substrate concentration to 200 g L\(^{-1}\) increased TPA yield (2.9, 3.5, and 4.9 %) with the reactor stirring speed enhancement (100, 400, and 800 rpm, respectively). For all experiments conducted under different stirring speeds, the best result was obtained for 220 g L\(^{-1}\) PC-PET under a stirring speed of 800 rpm that resulted in a TPA yield of 9.2 % after 96 h, which corresponds to 105.62 mmol L\(^{-1}\) of TPA released.

For the three series of experiments, the initial product formation rate goes through a maximum, corroborating the limited enzyme diffusion into substrate surface for PC-PET concentrations above this maximum. As the reactor stirring speed decreases, a thicker boundary layer around solid particle surfaces increases, resulting in a higher mass transfer resistance in the external film of the solid [41]. Such behavior was observed in the PET enzymatic hydrolysis conducted at 100 rpm, in which lower initial rates were noted for all substrate concentrations.

The PET enzymatic hydrolysis has similar aspects to the hydrolysis of other polymers as cellulose [24, 30]. Ingesson and collaborators [42] studied the enzymatic hydrolysis of cellulose and they reported that an increase of 7.5 % on the initial substrate concentration resulted in the reduction of the initial hydrolysis rate and the conversion yield for different shaking regimes (reaction flasks were shaken continuously or intermittently). The authors attributed this behavior to end-product inhibition and mass transfer limitations within the reaction mixture due to the high viscosity of the slurry (reaction medium).

Thus, considering that the best results were obtained for reactions carried out at 800 rpm stirring speed, further experiments were conducted with the same stirring speed to
minimize the diffusion restriction for the kinetic study and to evaluate the enzyme inhibition by hydrolysis products.

3.3. Effect of PC-PET concentration: Kinetic study

The enzymatic hydrolysis of PET is influenced by product inhibition [43]. The water-soluble hydrolysis products, MHET and BHET, are reported in the literature as competitive inhibitors of cutinases from *Thermobifida* species [30, 38, 43]. These products and PET compete for the active site responsible for the occurrence of the enzymatic reaction. Thus, some experiments were performed by supplying an initial amount of BHET, MHET, and TPA to the PC-PET hydrolysis medium reaction to analyze the product inhibition.

Figure 3 shows that the sum of the hydrolysis product concentration released from the PC-PET varied essentially linearly with the amount of TPA, MHET, or BHET added to the reaction medium. The lack of HiC inhibition by BHET, MHET, and TPA evidences an excellent feature for a future scale-up of a bioprocess using a single enzymatic system. This approach could reduce the cost and the complexity of reactor design or operation [44], once multi-enzyme systems could be limited concerning different optimal activity ranges and deactivation kinetics [45].

**Figure 3**

The kinetics of PC-PET hydrolysis catalyzed by HiC was studied by employing a heterogeneous model based on enzyme-limiting conditions proposed by Barth and collaborators [Barth, 2015]. Even though cutinases from *Thermobifida sp.* present a competitive inhibition effect by reaction products [30, 38, 43], the cutinase from *Humicola insolens* seems not to be inhibited at 70 °C by any of PET products, which suggests that the enzyme could be used in its maximum activity. Thus, by adjusting the model, the inhibitor contribution term ($\beta$) was considered equal to 0, since there was no inhibition contribution, and then, it was possible to determine the reaction constants through Equation 10.

The reaction rates were determined from the sum of released hydrolysis product concentrations in the reactions with different initial PET concentrations, and it was plotted as a function of substrate concentration for two PC-PET granulometric size ranges (Figure 4).
high determination coefficient ($R^2$) was obtained in the fitting to the kinetic model given by Equation 10, which was superior to 0.978 for both ranges, thereby confirming the model validity. The kinetic parameters were obtained by nonlinear regression analysis, and the results are presented in Table 1.

**Figure 4**

**Table 1**

The PC-PET fraction with the smaller particle size range ($F_1$) presented a larger specific area than $F_2$ (Table 1), which means a higher substrate surface area available. Therefore, the reaction carried out with the smallest PET particles showed a 2.4-fold higher hydrolysis rate constant ($k$) than the larger particle size range ($F_2$). This is expected, as the enzymatic PET hydrolysis occurs in a heterogeneous interface, and PET erosion depends on substrate surface availability. Similar behavior was also observed in other studies [46, 47], in which the decreasing particle size led to higher PET depolymerization due to increased surface area. Besides, comparable adsorption equilibrium constants ($K$) were obtained for both PC-PET particle size ranges by analyzing them based on initial substrate concentration. On the other hand, the value of $K$ (0.0051 mL cm$^{-2}$) estimated for the $F_1$ range, based on the PC-PET surface area, was 6.6-fold lower than that obtained for $F_2$ (0.0339 mL cm$^{-2}$), which is almost equal to the specific area ratio between $F_1$ and $F_2$. Therefore, it can be suggested that the accessibility of the degradable ester bonds for different substrate particle sizes was not significantly affected by enzyme affinity to PET, differently from what was seen when PET samples with different crystallinities were evaluated [40].

The proposed kinetic model was able to describe PC-PET erosion since this reaction depends on the substrate surface availability. Thus, different temperatures were also evaluated using this model to investigate this effect on enzymatic PET hydrolysis, which is relevant to take into account in bioreactor design and further scaling-up strategies to implement the enzymatic PET hydrolysis route.

3.4. **Effect of temperature**
It is well known that temperature significantly affects the hydrolysis of PC-PET [20, 38, 48]. Cutinases must be thermally stable (at temperatures above 60 °C) for the hydrolysis of semi-crystalline PET [33, 49–51]. Such behavior is necessary because the mobility of the polymer chain increases above the glass transition temperature \( (T_g) \), which for PC-PET occurs at 78.5 °C [52] but it may be lowered by approximately 10 °C in aqueous solutions [53, 54].

The temperature effect on the hydrolysis rate was addressed in this work through Arrhenius law in order to seek the best temperature range to use the HiC. Thus, several PC-PET concentrations were evaluated at each temperature (40, 50, 55, 60, 70, and 80 ºC), and the heterogeneous kinetic model was adjusted, as shown in Figure 5. An increase in the initial rate was observed with the rise of temperature, except at 80 ºC. The highest hydrolysis rate was observed at 70 ºC, closer to the \( T_g \) of PET, where chain mobility of the polymer is higher. Probably, the temperature of 80 ºC led to enzymatic denaturation, even though the literature reports that the optimum temperature range is between 75 and 80 ºC for the hydrolysis of PET using HiC [20, 32].

**Figure 5**

It can be seen from Figure 5 and Table 2 that the kinetic model was able to describe the data with a good statistical agreement for all temperatures studied, as indicates the \( R^2 \) values, that were higher than 0.949 in all cases. The worst adjustments were observed at the lowest temperatures (40 and 50 ºC), which can be attributed to the gap between the reaction temperature and the \( T_g \). The hydrolysis of PC-PET showed a 2.4-fold higher hydrolysis rate constant (k) and maintained a similar value of adsorption equilibrium constants (K) by increasing the reaction temperature from 60 ºC to 70 ºC. On the other hand, an extremely pronounced effect was observed by reducing the reaction temperature from 70 ºC to 55 ºC, diminishing the reaction rate by about 78.6%, which can be explained in the light of the low mobility of the polymer at 55 ºC. The adsorption constant (K) decreases moderately with the increase of temperature, as expected, indicating stronger enzyme adsorption at lower temperatures. These results show that the reaction rate constant (k) has a stronger influence on the overall reaction rate than the adsorption equilibrium constant (K), as reported by Basu and collaborators [55].

**Table 2**
The availability of thermodynamic reaction parameters is of interest in investigating the structure-reactivity relationship [37]. The enzymatic reaction starts with the initial binding of a substrate by an enzyme, and then, it proceeds by increasing their mutual affinity in water, which leads to a rate enhancement [56]. The enzyme binding interactions include fixation of the reacting groups of the substrate at the active site in the correct position and a destabilization of the ground state that brings a considerable loss of entropy, thus decreasing the activation energy of the reaction [57]. As the enzyme conformation changes during the catalytic event, it would be useful to study structures approaching the transition state of the biocatalyzed reaction. The transition state itself is too short-lived, but an indirect approach, such as Pauling’s transition state stabilization theory, may provide a partial solution to this problem [58]. This theory proposes that enzymes are developed to bind tightly to the transition state rather than the reactant or product. The high affinity of the enzyme active site by transition state results in lowering the activation state.

An exponential approach was adopted to determine the activation parameters, and the results are shown in Figure 6 and Table 3. The activation energy (\(E_a\)) and Arrhenius constant (\(A_0\)) were obtained by the Arrhenius equation (Equation 11) through estimated values of the reaction rate constant, and the correlation coefficient of the nonlinear regression was 0.998. The excellent fit of the data to the Arrhenius equation confirms that the kinetic model, estimated from different substrate concentrations, was adequate to describe the enzymatic hydrolysis of PET. The apparent activation energy found for PC-PET hydrolysis using HiC was 98.9 ± 2.6 kJ mol\(^{-1}\). This \(E_a\) value obtained for enzymatic hydrolysis of PC-PET has the same order of magnitude as other chemical catalytic routes. Carta and collaborators [59] evaluated the recycling of PET through neutral hydrolysis by fitting an autocatalytic model of half-order, and they found activation energy of 123 kJ mol\(^{-1}\), while the PET hydrolysis using a dual functional phase transfer catalyst resulted in lower activation energy (63 kJ mol\(^{-1}\)) at a temperature range of 115-145 °C [60]. The literature reports similar values of activation energy for acid-hydrolysis (100-110 kJ mol\(^{-1}\)) [61, 62] and neutral hydrolysis (90-123 kJ mol\(^{-1}\)) of PET [63, 64].

Two contributions can describe the overall activation free energy (\(\Delta G^\# = \Delta H^\# - T\Delta S^\#\)), one referring to the entropy of activation (-\(T\Delta S^\#\)) while the other to the heat one (\(\Delta H^\#\)) [Aqvist, 2017]. Thus, the estimation of both parameters was performed using the Eyring-Polonyi equation (Equation 12) rate, and the values estimated for \(\Delta H^\#\) and \(\Delta S^\#\) were equal to 96.1 ± 3.6 kJ mol\(^{-1}\) and 10.8 J ± 9.8 mol\(^{-1}\) K\(^{-1}\), respectively. Wolfenden and collaborators [65] observed that \(\Delta S^\#\) is low for hydrolytic reactions, and on the other hand, \(\Delta H^\#\) is high and positive for
slow reactions that are catalyzed by enzymes. Besides, the entropy contribution (-TΔS°) implies that the degree of randomness decreases with the adsorption [55] of the enzyme. As both phenomena occur simultaneously, the adsorption step is followed by a considerable release of enthalpy, which results from the increase in substrate affinity by the enzyme [56].

The Circe effect hypothesis postulates that enzymes spend some part of the binding free energy on binding tightly to the substrate [57]. Thus, the reaction progress, that is, the ES complex shifting from the ground state to the transition state, is mainly enthalpic in its origin [56]. It was reported that enthalpy tends to dominate the enzyme thermodynamics on reactions involving single substrates and hydrolytic reactions in which water is present in abundance [65]. Therefore, the activation barrier can be climbed without any entropy loss, producing an increase in the enzymatic reaction rate [66].

**Figure 6**

**Table 3**

Thus, the estimation of thermodynamic activation parameters for PC-PET hydrolysis allowed the understanding of the origin of enthalpic contributions to enzymatic rate enhancement. This comprehension will be useful for rationalizing further enzymological experiments on PC-PET hydrolysis and enzyme (HiC) engineering and design.

### 4. Conclusion

The kinetic study of post-consumer PET (PC-PET) hydrolysis was performed based on enzyme-limiting conditions under a stirring speed of 800 rpm to avoid diffusion restriction, based on preliminary obtained data. The inhibition effect of HiC by the main PET hydrolysis products (TPA, MHET, and BHET) was evaluated at 70 °C, and no inhibition evidence was observed, which suggests that the potential of the enzyme can be used at its maximum. This represents a promising aspect for future evaluations since there is no necessity of incorporating strategies to circumvent inhibition problems, such as the employment of a multi-enzymatic system. The excellent agreement between experimental data and a model based on enzyme-limiting conditions validates its use for describing the enzymatic hydrolysis of PC-PET with different granulometric size ranges and temperatures. The reaction rate constant increased by an exponential factor with the reaction temperature enhancement, except at 80 °C, which may
have been caused by enzymatic denaturation. The activation energy was determined through the Arrhenius equation with an excellent adjustment ($R^2 = 0.998$), and the value obtained for enzymatic hydrolysis had the same order of magnitude as those parameters reported in the literature for chemical PET hydrolysis. Thermodynamic parameters obtained for enzymatic PC-PET hydrolysis were found to have an activation enthalpy of $96.1 \pm 3.6$ kJ mol$^{-1}$ and activation entropy of $10.8 \pm 9.8$ J mol$^{-1}$ K$^{-1}$. Kinetic correlations provided a reliable mathematical model of the heterogeneous hydrolysis of PC-PET using HiC as biocatalyst, providing a significant amount (105.62 mmol L$^{-1}$) of released TPA, which can benefit the implementation of a circular economy.

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6. Reference

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Figure 1: Time course of enzymatic hydrolysis of post-consumer poly(ethylene terephthalate) (PC-PET) for different substrate concentrations. Reactions were carried out at 70 °C with PC-PET particle size range of 0.075-0.250 mm, 200 mmol L⁻¹ sodium phosphate buffer (pH 7.0), and 1.0 mgₚrotein mL⁻¹ of *Humicola insolens* cutinase (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Figure 2: Initial post-consumer poly(ethylene terephthalate) (PC-PET) hydrolysis rate for different reactor stirring speeds: 100 (closed square), 400 (open circle), and 800 rpm (closed triangle). Reactions were carried out at 70 °C, using PC-PET with the particle size range of 0.075-0.250 mm, 200 mmol L⁻¹ sodium phosphate buffer (pH 7.0), and 1.0 mgₚrotein mL⁻¹ of biocatalyst.

Figure 3: Molar concentration of products obtained from post-consumer poly(ethylene terephthalate) (PC-PET) hydrolysis in different initial concentrations of terephthalic acid (TPA), mono(2-hydroxyethyl) terephthalate (MHET), and bis(2-hydroxyethyl) terephthalate (BHET). Reactions were carried out at 70 °C, in sodium phosphate buffer 200 mmol L⁻¹ (pH 7.0), 1.0 mgₚrotein mL⁻¹ of HiC, 50 g L⁻¹ of PC-PET with a particle size range of 0.250-0.600 mm, under 800 rpm stirring speed, the effect of the addition of TPA and MHET was evaluated after 96 h, and for BHET, it was analyzed after 24 h.

Figure 4: Adjustment of the heterogeneous kinetic model for post-consumer poly(ethylene terephthalate) (PC-PET) hydrolysis by *Humicola insolens* cutinase (HiC) using different substrate concentrations and two PC-PET granulometric size ranges: 0.075-0.250 mm (F1, squares) and 0.250-0.600 (F2, triangles). Reactions were carried out at 70 °C, 200 mmol L⁻¹ sodium phosphate buffer (pH 7.0), 1.0 mgₚrotein mL⁻¹ of HiC, under 800 rpm stirring speed.

Figure 5: Effect of temperature and substrate concentration on the initial rate of for post-consumer poly(ethylene terephthalate) (PC-PET) hydrolysis and adjustment of the kinetic
model (solid line). Reactions were carried out under 800 rpm stirring speed, 200 mmol L\(^{-1}\) sodium phosphate buffer (pH 7.0), 1.0 mg\text{protein mL}^{-1} of *Humicola insolens* cutinase (HiC), and PC-PET with the particle size range of 0.075-0.250 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Figure 6: Arrhenius plot for post-consumer poly(ethylene terephthalate) (PC-PET) hydrolysis using *Humicola insolens* cutinase (HiC). The kinetic parameters were estimated based on enzyme-limiting conditions.
Table 1: The estimated parameter for the kinetic model for post-consumer poly(ethylene terephthalate) (PC-PET) hydrolysis by *Humincola insolens* cutinase (HiC) for two substrate granulometric size ranges: 0.075-0.250 mm (F1, squares) and 0.250-0.600 (F2, triangles). Reactions were carried out at 70 °C, 200 mmol L\(^{-1}\) sodium phosphate buffer (pH 7.0), 1.0 mg\textsubscript{protein} mL\(^{-1}\) of HiC, under 800 rpm stirring speed.

Table 2: Kinetic parameters of enzymatic post-consumer poly(ethylene terephthalate) (PC-PET) hydrolysis model for different temperatures. The reactions were performed under 800 rpm stirring speed, phosphate buffer 200 mmol L\(^{-1}\) (pH 7.0), *Humincola insolens* cutinase (HiC) concentration of 1.0 mg\textsubscript{protein} mL\(^{-1}\), and PC-PET with a particle size range 0.075-0.250 mm.

Table 3: Activation parameters of enzymatic post-consumer poly(ethylene terephthalate) (PC-PET) hydrolysis model. The kinetic parameters used on Arrhenius and Eyring-Polanyi equations were estimated based on enzyme-limiting conditions.