Simple Kinetic Model for the Heterogeneous Enzymatic Hydrolysis of Natural Poly(3-hydroxybutyrate)

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ABSTRACT: The kinetics of the enzymatic degradation of bacterial poly(3-hydroxybutyrate) (PHB) is studied using PHB-depolymerase A from *Pseudomonas lemoignei* (Tris−HCl buffer, pH = 8, T = 37 °C). Biodegradation experiments are performed on PHB in the form of both compression-molded films and powder suspension. From WAXS and DSC measurements the two substrates show the same crystalline fraction. The rate of hydrolysis of PHB films is determined by gravimetry and also through spectrophotometric quantification of the hydrolysis products at λ = 210 nm. For the suspension of PHB particles, a turbidimetric determination of the biodegradation rate is applied. A simple two-step kinetic model is proposed, which predicts that the hydrolysis rate per unit substrate surface area reaches a plateau at high enzyme concentrations. The model satisfactorily describes the enzymatic degradation results of PHB film and PHB powder suspension, provided that the remarkable changes of exposed area caused by enzymatic attack to the latter substrate are taken into account. Analysis of the enzymatic degradation results yields analogous hydrolysis rate constants for film (1.48 μg cm^{-2} min^{-1}) and powder suspension (1.42 μg cm^{-2} min^{-1}).

Introduction

Poly(3-hydroxybutyrate), PHB, is accumulated by a number of bacteria as intracellular carbon and energy reserve material.[1] According to the metabolic needs of the microorganism, the polymer is degraded to low molecular weight compounds by intracellular enzymes (PHB-depolymerases). In addition, when PHB is present in the environment, many microorganisms (bacteria, fungi, and algae)—independent of the ability to synthesize PHB themselves—secrete extracellular PHB-depolymerases in order to degrade the polyester and use the biodegradation products as nutrients.[2]

The enzymatic hydrolysis of extracellular PHB has been investigated by several groups[3−7] according to the classical Michaelis−Menten kinetic model. Mukai et al.[8] first called attention to the fact that enzymatic hydrolysis of solid PHB is a heterogeneous catalytic reaction, which cannot be appropriately described by a kinetic model originally proposed for homogeneous-phase enzymatic reactions. Hence a new kinetic model was suggested[8] in order to account not only for heterogeneity of the process but also for the different role played by the binding and active sites of the enzyme[9−12] in the different steps of the enzymatic reaction. In Mukai's model the initial equilibrium absorption reaction of PHB-depolymerase on the solid substrate specifically regards the enzyme binding domain. Binding is followed by the hydrolysis reaction, which involves the enzyme catalytic domain and the fraction of substrate surface not occupied by bound enzyme ("free" surface). The model predicts that, with increasing enzyme concentration (C_e), the rate of hydrolysis increases to a maximum and then decreases at high C_e, owing to surface overcrowding by bound enzyme and consequent lack of "free" substrate surface, which is required as a reactant in the hydrolysis reaction.

Recently, Mukai's model was further modified by Timmins et al.[13] in order to include the effect of substrate concentration on the rate of heterogeneous degradation of PHB.

The aim of this paper is to demonstrate that enzymatic degradation of bacterial PHB can be satisfactorily described by a quite simple two-step kinetic model, which is formally analogous to that of Michaelis and Menten but takes into account the solid nature of the substrate. The main difference with respect to the mentioned models by other authors[8,13] is that the enzyme−substrate complex formed in the first reaction step (heterogeneous equilibrium absorption of enzyme onto the solid substrate) directly undergoes the hydrolysis reaction (second step) without involving the "free" substrate surface as an additional reactant.

The kinetic model proposed is successfully applied to the enzymatic degradation of bacterial PHB, in both film and powder form, using PHB-depolymerase A from *Pseudomonas lemoignei*.

Experimental Section

Materials. Bacterial poly(3-hydroxybutyrate), PHB, was an ICI product (GO8, $M_w = 5.39 \times 10^5$, $M_w/M_n = 4.11$) provided in powder form.

PHB films (thickness 0.2−0.3 mm) were obtained by compression molding the "as received" powder between two Teflon plates containing a spacer, at 195 °C for 1 min under a pressure of 2 ton/m^2 (Carver C12 laboratory press). The compression-molded PHB films were allowed to age at room temperature for at least 3 weeks before use in order to reach equilibrium crystallinity.

A suspension of the "as received" PHB powder was prepared as follows: PHB was suspended (0.3% w/v) in 50 mM Tris−HCl buffer (pH = 8.0, 1 mM $CaCl_2$) and subjected to ultrasonic treatment (Transsonic T460 Elma 35 kHz) for 10 min before use.

Biodegradation Measurements. Biodegradation experiments were carried out by means of PHB-depolymerase A from *P. lemoignei* purified as previously described.[14] Measurements were performed at 37 ± 0.1 °C in Tris−HCl buffer (pH = 8.0) containing $CaCl_2$ (1 mM) as enzyme stabilizer.

Films. Two procedures were adopted to follow the hydrolysis of PHB films.

(1) Rectangular film samples (size: 12 × 8 mm^2, 20 × 7 mm^2, 25 × 8 mm^2) were placed in thermostated vials containing 1.5

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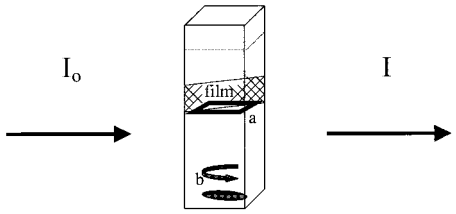

Figure 1. Experimental setting for the spectrophotometric determination of the enzymatic hydrolysis products of PHB films: (a) silicon rubber frame; (b) magnetic stirrer.

mL of buffer (50 mM), and an appropriate amount of enzyme was injected (enzyme concentration range: $C_e = 0-14\ \mu$g/mL). After 20 h the samples were removed from enzymatic solution, washed with distilled water, and dried under vacuum to a constant weight (analytical electronic Sartorius RC210D balance; reproducibility ±0.02 mg). Weight loss was used to quantify the extent of biodegradation. Each measurement was run in duplicate, and the weight loss was averaged.

(2) A modification of the spectrophotometric method proposed by Mukai et al.[8] was applied to PHB films (12×8 mm^2). The experimental setting is illustrated in Figure 1: a small silicon rubber frame was inserted in the quartz cuvette in order to locate the film along the diagonal of the cuvette section (ensuring optimal access of the enzyme to both film sides) and above the light beam path (no interference with the UV radiation); the magnetic stirrer at the cuvette bottom provided efficient transport of the reaction products toward the light beam. A Varian Cary-1 spectrophotometer was used to measure the absorbance at $\lambda = 210$ nm as a function of time. The cuvette contained 2 mL of buffer (10 mM, in order to keep buffer absorption low), and the reaction was started by enzyme injection. To subtract from the measured absorbance all spurious contributions (such as absorbance from nonenzymatic hydrolysis products), the reference cuvette contained buffer and a PHB film in the same setting as the reaction cuvette (Figure 1). The experiments were stopped after 3 h, and the pH of the reaction cuvette was checked. At the end of all biodegradation measurements the pH did not change, showing that over the whole range of C_e used 10 mM buffer concentration was sufficient to neutralize the hydroxybutyric acid developed in the hydrolysis reaction. The concentration of HB units liberated by PHB films during enzymatic hydrolysis was calculated from the measured absorbance at $\lambda = 210$ nm, using the extinction coefficient of HB (75 cm^{-1} M^{-1}) experimentally determined for 3-hydroxybutyric acid (Aldrich). Reliability of the spectrophotometric quantification of the total amount of HB liberated by enzymatic hydrolysis during the test was checked by weight loss measurements at the end of the experiments, obtaining an excellent agreement (>95%).

In the kinetic treatment of all biodegradation results obtained using PHB films, the surface area exposed to the enzymatic solution was taken as the sum of the two film sides, disregarding the lateral area in the thickness direction. This approximation implies a maximum 5% underestimation of the area with the thickest films used. All films recovered after enzymatic hydrolysis retained their original rectangular shape and size and showed no holes at optical microscope resolution (×320). Therefore, with the above approximation regarding thickness, the exposed film area was taken as constant during the course of the biodegradation experiments.

Powder Suspension. Biodegradation experiments were performed using a Varian Cary-1 spectrophotometer (cuvettes provided with magnetic stirring), to measure the decrease of optical density of the suspension at $\lambda = 650$ nm (OD_{650}) during enzymatic hydrolysis of PHB particles. The PHB suspension (120 μg/mL) was characterized by a stable OD_{650} value (0.8). After enzyme addition, the hydrolysis and solubilization of PHB granules caused a decrease of the number of particles in suspension, that scattered the radiation, hence a decrease of OD_{650}. From the slope of the linear portion of the OD curve as a function of time, the rate of enzymatic hydrolysis was calculated (units: OD_{650} min^{-1}). On the basis of the linear correlation obtained between OD_{650} (in the range 0−1.4) and PHB suspension concentration, the hydrolysis rate values were converted from turbidimetric into mass units (mg min^{-1}).

Table 1. Melting Properties and Crystallinity Degree (χ_c) of PHB Substrates

sample	T_m (°C)	ΔH_m (J/g)	χ_c (DSC)[a]	χ_c (WAXS)[b]
film	174	98	0.67	0.60
powder	173	99	0.68	0.62

[a] ±0.02. [b] ±0.05.

Experimental Techniques. Differential scanning calorimetry (DSC) was performed with a DuPont 9900 thermal analyzer in the temperature range −80 to +220 °C at a heating rate of 10 °C/min.

Wide-angle X-ray diffraction measurements (WAXS) were carried out with a Philips PW 1050/81 diffractometer controlled by a PW 1710 unit, using nickel-filtered Cu Kα radiation ($\lambda = 0.1542$ nm, 40 kV, 30 mA). The crystallinity degree was calculated through graphical integration of the diffracted intensity data in the 2θ range 10°− 60° and subtraction of the amorphous scattering band intensity.

A scanning electron microscope (SEM) (Philips 515) was used to observe PHB powder particles, after sputter-coating with gold.

Results

Substrate Characterization. PHB, in both film and powder form, was characterized by DSC measurements. Table 1 reports the crystallinity content calculated from the experimental melting enthalpy ΔH_m and from the literature $\Delta H_m°$ value for 100% crystalline PHB.[15] Crystallinity of the two PHB substrates was also estimated from WAXS measurements (Table 1). Both techniques yielded the same crystalline fraction for PHB film and powder, the value obtained from DSC being slightly higher than that from WAXS, as is commonly found.

The total surface area (A) exposed to the enzymatic solution by the suspension of PHB particles was obtained through the following equation:

$$A = (W/w)A_p \qquad (1)$$

where W and w are total PHB mass and single particle mass, respectively, and A_p is the surface area of a single particle. A_p was estimated on the basis of SEM evidence that PHB particles were essentially smooth spheres with an average diameter of 1 μm. The mass of a single particle (w) was calculated from the experimental average particle volume, assuming a crystalline/amorphous ratio 60/40 (Table 1) and using literature data[15] for crystalline and amorphous PHB density. From eq 1 the surface area of the PHB powder suspension used in the enzymatic degradation experiments was 12 cm^2.

Enzymatic Degradation of PHB Films. Figure 2 shows the rate of enzymatic hydrolysis (weight loss after 20 h of exposure) of PHB films as a function of surface area, at constant enzyme concentration ($C_e = 0.056\ \mu$g/mL). When the area is about 2.5 cm^2, the rate reaches a plateau, insensitive to further area increments. The behavior observed is reminiscent of the typical dependence of rate on substrate concentration of enzyme-catalyzed reactions that follow simple Michaelis−Menten kinetics. The attainment of a constant rate in Figure 2, illustrates the limiting role played in these experiments by the enzyme concentration employed.

Conversely, experiments where film area was kept constant and enzyme concentration changed, showed that the rate of PHB hydrolysis was limited by the area

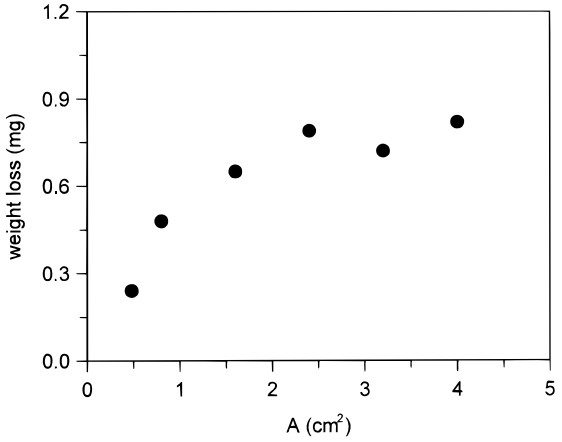

Figure 2. Weight loss of PHB films, after 20 h of exposure to PHB-depolymerase solution ($C_e = 0.056$ μg/mL), as a function of film surface area (A).

of substrate exposed to enzymatic solution. Figure 3 collects rate results obtained on PHB films with three different surface areas ($A_1 = 1.92$ cm^2, $A_2 = 2.80$ cm^2, $A_3 = 4.00$ cm^2) as a function of enzyme concentration. At constant film area, the hydrolysis rate dependence on C_e changes from first to zero-order kinetics. The maximum rate reached by each set of PHB films (plateau value) increases with the surface area of substrate exposed to enzymatic solution. This observation suggests we attempt a normalization of the values of Figure 3, using film area as the normalizing factor: the ratio of each experimental rate result to the corresponding area yields the plot of Figure 4. All "normalized" rate data fall on a single hyperbolic curve as a function of enzyme concentration, demonstrating that the rate of enzymatic hydrolysis of PHB films directly depends on the exposed surface area. In keeping with this finding, the kinetic model proposed in this work predicts direct proportionality between the rate of enzymatic degradation of PHB and surface area of the solid substrate.

The kinetic model is very simple and is formally analogous to that of Michaelis and Menten:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \tag{2}$$

$$ES \xrightarrow{k_2} P + E \tag{3}$$

where E and S are enzyme and substrate, respectively, ES is the enzyme/substrate complex, and P represents hydrolysis reaction products.

The equilibrium adsorption reaction (2) involves a solid substrate and can be expressed as

$$k_1 C_e A(1 - \vartheta) = k_{-1} A\vartheta \tag{4}$$

where A is the substrate surface area, ϑ is the fraction of substrate surface occupied by the ES complex, $(1 - \vartheta)$ is the "free" surface fraction, and C_e is the initial enzyme concentration.

From eq 4 the ϑ parameter is derived:

$$\vartheta = KC_e/(1 + KC_e) \tag{5}$$

where $K = k_1/k_{-1}$ is the adsorption equilibrium constant.

For the hydrolysis reaction (3), the proposed model gives the following rate equation:

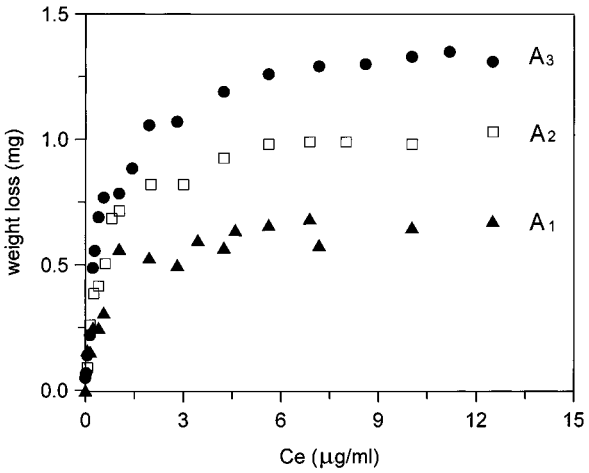

Figure 3. Weight loss of PHB films, after 20 h of exposure to PHB-depolymerase solution, as a function of enzyme concentration (C_e). Film surface area: $A_1 = 1.92$ cm^2; $A_2 = 2.80$ cm^2; $A_3 = 4.00$ cm^2.

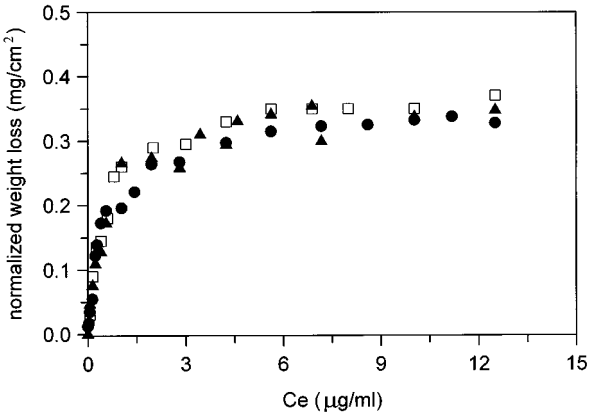

Figure 4. Weight loss per unit surface area of PHB films (area: ▲ = 1.92 cm^2; □ = 2.80 cm^2; ● = 4.00 cm^2), as a function of enzyme concentration (C_e).

$$V = k_2 A\vartheta = k_2 A[KC_e/(1 + KC_e)] \tag{6}$$

whose linear form

$$A/V = (1/Kk_2)(1/C_e) + 1/k_2 \tag{7}$$

can be used to easily derive adsorption (K) and hydrolysis rate (k_2) constants from the slope and intercept of the straight line fit to the experimental rate results.

To test the validity of the model in the case of PHB films, instead of using the results of Figures 2−4 (single weight loss measurements after a rather long exposure time, 20 h), a new set of experimental data was collected by the spectrophotometric method illustrated in Figure 1, which quantifies (from their absorbance at $\lambda = 210$ nm) the hydrolysis products released from the films as a function of time.

The results are plotted in Figure 5a as the rate of hydrolysis vs enzyme concentration, and in Figure 5b in the reciprocal form suggested by eq 7. The line drawn in Figure 5b is the data linear regression, whose slope and intercept yield the kinetic constants K and k_2 (Table 2). Introduction of K and k_2 in eq 6 allows calculation of the enzymatic hydrolysis rate of PHB films of the given size (A) as a function of enzyme concentration, according to the model. The calculated curve, drawn in Figure 5a, very satisfactorily fits the experimental

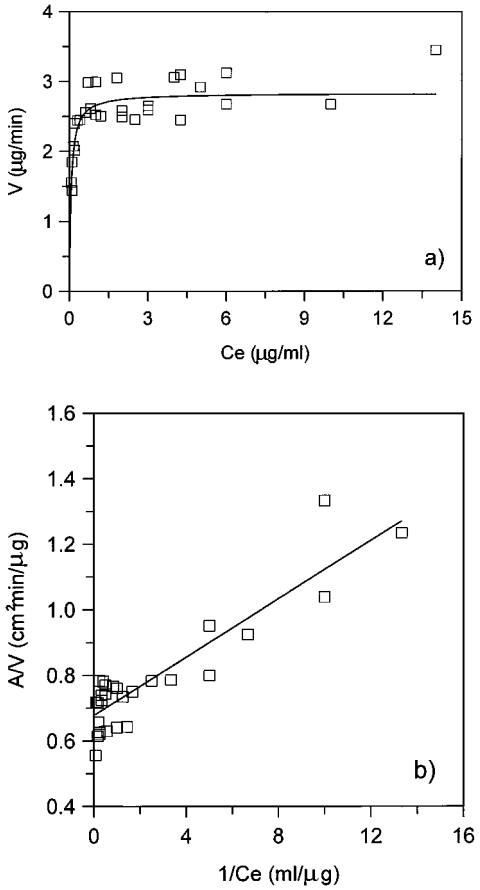

Figure 5. (a) Rate (V) of enzymatic hydrolysis of PHB films ($A = 1.92$ cm^2) by the spectrophotometric method (see text and Figure 1), as a function of enzyme concentration (C_e). The curve is the rate calculated according to the model proposed in this paper (eq 6). (b) Same results plotted according to eq 7. The line is the least-squares fit to the experimental data.

Table 2. Equilibrium Adsorption Constant (K) and Rate Constant (k_2) of PHB Enzymatic Hydrolysis

sample	K (mL/μg)	k_2 (μg/cm^{2}min)
film	15 ± 2	1.48 ± 0.04
powder suspension	8 ± 2	1.42 ± 0.04

rate results, demonstrating that the model proposed in this work successfully describes the kinetics of enzymatic degradation of PHB films by depolymerase A from *P. lemoignei*.

Enzymatic Degradation of PHB Powder Suspension. The rate of enzymatic hydrolysis of the suspension as a function of enzyme concentration is shown in Figure 6, together with the results on PHB films illustrated above (for the sake of comparison). A behavior strikingly different from that of the films is shown by the suspension of PHB particles: with increasing C_e, the rate increases to a maximum and then decreases, instead of leveling off. Moreover, the maximum hydrolysis rate (V_{max}) reached by the suspension is much higher than that of the film.

With respect to the latter observation, the hypothesis that the large V_{max} discrepancy might arise from relevant morphological differences in the two substrates, based on the fact that in PHB films the rate of enzymatic erosion increases with a decrease of crystallinity,[16] is easily ruled out by the results of the thermal and structural characterization, which showed that film and powder have the same crystallinity degree (Table 1).

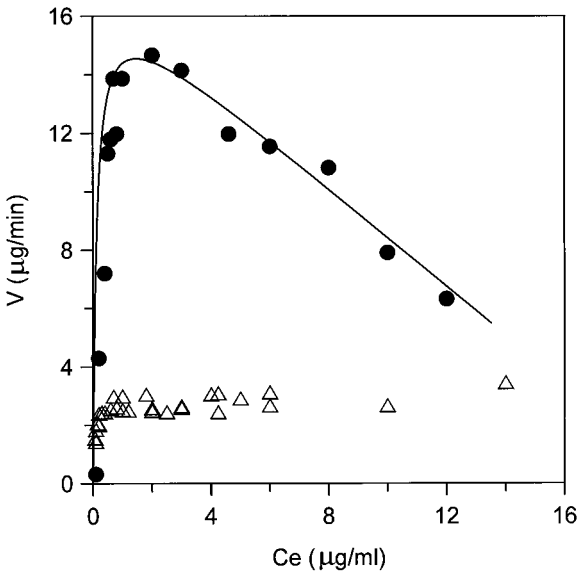

Figure 6. Rate (V) of enzymatic hydrolysis of PHB particle suspension (●) and film (Δ, same data as in Figure 5) as a function of C_e. The curve is the hydrolysis rate of the suspension, calculated according to the model proposed in this paper (eq 6).

To explain the V_{max} difference, it is important to recall the effect of surface area on the maximum hydrolysis rate of PHB films previously discussed (Figure 3). Noteworthy, the surface area of film and suspension (1.92 and 12 cm^2, respectively) show approximately the same ratio (1:6) as the maximum rates plotted in Figure 6. This observation suggests that the high V_{max} of the PHB powder suspension is correlated with the large surface area exposed to PHB-depolymerase attack by this particular substrate. It is reasonable, therefore, to associate the V_{max} difference between film and powder (Figure 6) primarily with the area parameter.

When the hydrolysis rate results of Figure 6 are divided by the initial surface area of each substrate, the "normalized" rate of film and suspension practically overlap up to V_{max}. Obviously, at enzyme concentrations higher than that corresponding to V_{max}, the "normalized" hydrolysis rates of the two substrates maintain divergent trends, the rate of the suspension almost linearly decreasing with C_e, instead of leveling off at V_{max} as the film does.

The key to explaining the different behavior of the film and powder suspension at high enzyme concentrations may be sought again in the surface area of the substrate. The surface area exposed is a very crucial parameter, which should be carefully evaluated, especially when enzymatic hydrolysis results deriving from turbidimetric measurements are discussed. In fact, whereas during hydrolysis of PHB films no macroscopic substrate area changes came into play over the time scale of the experiments (as mentioned in the Experimental Section, films maintained their overall shape and only underwent slight thinning), in the peculiar measurements applied to the suspension a decrease of substrate surface area exposed to enzymatic solution is inherent in the method (the observed OD_{650} decrement reflects the decrease of the number of scattering particles caused by hydrolysis and solubilization of PHB). No OD_{650} change would be observed if the surface area (i.e., the number of particles) remained constant.

An area decrement is then intrinsically associated with the turbidimetric method employed. Such a phe-

nomenon clearly occurs at all enzyme concentrations used, including the low-C_e range. However, when C_e is low, the substrate area available in the suspension is so much in excess with respect to the enzyme present in solution that surface area reduction (slow at low C_e) does not significantly affect the measured hydrolysis rate. In other words, during the short time scale of the experiment, C_e remains far below the amount required to saturate the available (decreasing) surface. Consequently, in the low-C_e range the measured hydrolysis rate of the suspension increases with enzyme concentration, as would be expected in the absence of area changes (as mentioned above, the "normalized" rate values match those of the films), and reaches a V_{max} that correlates with the initial suspension surface.

On the contrary, when enzyme concentration falls in the range of saturation of the original suspension surface, any decrease of the latter caused by PHB hydrolysis directly influences the measured rate. The importance of this effect is expected to increase with C_e. In fact, at high enzyme concentrations the almost instantaneous decrease of substrate area caused by massive enzymatic attack to the particles inevitably yields measured OD decrements (i.e., degradation rates) sensibly lower than expected in the case of constant exposed area. The problem lays in the different time scales of the turbidimetric determination and of the extremely fast enzymatic attack at high C_e's. It is reasonable to suggest that if the real "initial" hydrolysis rate of the suspension could be experimentally determined, it should reflect the enzymatic attack on the original suspension surface ($A = 12$ cm^2) and a constant rate equal to V_{max} would be found at high enzyme concentrations.

Actually, in Figure 6 the experimental rate of the suspension in the high-C_e range is seen to decrease linearly with enzyme concentration. On the assumption that this behavior was essentially due to a rapid preliminary reduction of available surface, from the results of Figure 6 the following expression for the area "effectively" exposed to enzymatic attack during the turbidimetric measurement at each enzyme concentration was derived:

$$A_{eff}\ (\text{cm}^2) = 12(1 - 0.05C_e) \qquad (8)$$

The rate of hydrolysis of the suspension was then calculated according to eq 6, where A was substituted with A_{eff} (eq 8) and the kinetic constants K and k_2 (reported in Table 2) were obtained as described above (eq 7). The hydrolysis rate calculated from eq 6 is drawn as a continuous line in Figure 6. The agreement between model prediction and experimental results is surprisingly good, showing that the kinetic model proposed above to describe the enzymatic hydrolysis of PHB films can be successfully applied also to enzymatic degradation of a suspension of PHB particles, provided that a convenient expression for the exposed substrate surface is used.

Table 2 compares the kinetic constants of the two PHB substrates investigated in this work and shows that film and powder have the same hydrolysis rate constant k_2 and an equilibrium adsorption constant K that lies within the same order of magnitude. It is all but surprising that the two substrates show the same value of k_2, a parameter that represents the maximum rate of enzymatic hydrolysis per unit substrate area obtainable in defined experimental conditions with the given PHB-depolymerase. The two substrates were in fact constituted of PHB chains with the same molecular characteristics (the powder used in the suspension was also the starting material from which films were compression molded) and showed the same amorphous/crystalline ratio (Table 1).

Discussion

The aim of this paper was to describe by the same simple model the kinetics of enzymatic hydrolysis of bacterial PHB in both film and granular form. In the foregoing data analysis only macroscopic changes of the overall exposed area such as those occurring during enzymatic degradation of small particles in suspension were taken into account. It is, however, easily understood that more subtle surface changes associated with the selectivity of enzymatic attack inevitably occur during PHB enzymatic hydrolysis. Scanning electron microscopy of the surface of films before and after biodegradation has extensively documented[14,17] that preferential hydrolysis of the amorphous PHB chains changes the originally smooth surface into a rougher one, from which PHB crystals (that degrade at a slower rate than the amorphous phase) protrude. Additional evidence of preferential degradation of the amorphous phase was very recently obtained[18] using the ^{1}H NMR imaging technique as a tool to quantify the amorphous PHB content during enzymatic hydrolysis of PHB films.

Although in the present kinetic treatment morphology-dependent surface area changes have been intentionally neglected for the sake of simplicity, the results of this work demonstrate that it is possible to adequately describe the experimental enzymatic hydrolysis data by simply taking into account the original sample area and its macroscopic changes during the enzymatic degradation process.

The kinetic model proposed predicts the attainment of a plateau of the rate of enzymatic hydrolysis per unit substrate area at high enzyme concentration. An analogous behavior was reported some years ago for the rate of enzymatic degradation of cellulose[19] in one of the few papers, to our knowledge, that shows experimental rate vs C_e results for enzymatic hydrolysis reactions on solid substrates. Sattler et al.[19] studied the effect of enzyme concentration on the rate of cellulose hydrolysis, using different enzymes (cellulases) and substrates (cellulose obtained from various sources). In all enzyme−substrate combinations investigated, the rate of cellulose hydrolysis was reported to change as a function of enzyme concentration in the same way as presently found for PHB. Linear double reciprocal plots analogous to that shown for PHB in this work (Figure 5b) were also reported by Sattler et al. for the enzymatic degradation of the polysaccharide. Notwithstanding the known complexity of the enzymatic degradation process in cellulose, it is remarkable that the polysaccharide and PHB show such similar biodegradation behavior. This fact suggests that a hydrolysis rate that levels off at high C_e, as found in this work for PHB, may be a rather common feature in the enzymatic hydrolysis of solid substrates.

In this connection, it is important to recall earlier results on PHB by other authors,[8,13,20] where the hydrolysis rate was reported to show a more or less pronounced maximum as a function of C_e instead of reaching a plateau. To explain such findings, complex

kinetic models[8,13] were invoked, whose main difference from the model proposed in this paper was the presence of the substrate as reactant in both the adsorption and hydrolysis reaction steps. In light of the present results it seems reasonable to suggest that in the studies where PHB powder suspension was used as the substrate,[13] the rate decrement observed at high C_e, reported to be more evident at a lower initial suspension concentration (i.e., at smaller initial substrate area), was caused by a decrease of exposed surface, as argued above for the present PHB suspension results.

On the other hand, the literature data showing a maximum in the rate vs C_e dependence using PHB films as substrate[8,20] are clearly in striking contrast to the large body of evidence on the enzymatic degradation of PHB films collected by the present authors both in this and in previous work.[14] The most plausible explanation for the rate decrease at high C_e observed in the cited studies on PHB films[8,20] is that the effectively exposed surface area at high C_e was, for some reason, sensibly lower than the original film area. The reason for the occurrence of surface changes might be sought in the very small thickness of the films employed in the mentioned investigations (0.04−0.07 mm compared with 0.2−0.3 mm of the films used in this work) and in the rather long exposure times considered (18−19 h), which caused weight losses as high as 60%[8] or even 80%.[20] It is likely that such an extensive erosion of very thin films produced small holes or other shape changes leading to a decrease of the effective surface area and hence of the measured hydrolysis rate.

As regards the rate vs C_e behavior to be expected in the case of PHB films, it is worth noting that in a recent work by Doi's group[21] it is suggested that the hydrolysis rate of films should be proportional to the concentration of adsorbed enzyme, expressed by the Langmuir equation. A direct consequence of such a proportionality, not mentioned in the cited paper, is that the hydrolysis rate should then depend on C_e as predicted by the Langmuir adsorption isotherm, i.e., with an expression analogous to eq 6 of this work. This fact is in clear contrast to the rate vs C_e behaviors showing maxima, which were repeatedly reported by the same authors[8,22−25] in earlier studies and were the basis for the model proposed by Mukai et al.,[8] but is perfectly in line with the findings of the present paper and calls for a general reconsideration of the exposed substrate area issue.

We may conclude that, although the simple model proposed in this paper satisfactorily describes the experimental PHB enzymatic degradation results, there are a number of problems that remain open for discussion and deserve further consideration. Among others, attention is called to the area changes associated with selective enzymatic degradation of the substrate surface, which need adequate quantification and should be taken into account in a comprehensive description of the mechanism of enzymatic hydrolysis of the polyester. A second important and still controversial point[16,18] regards the different rate of hydrolysis of PHB in the amorphous and in the crystalline state. Further experimental work is needed in order to elucidate the interplay of the mentioned factors (exposed area and amorphous vs crystalline hydrolysis rate) in the enzymatic degradation process of PHB. Along these lines in a forthcoming paper we will discuss the role of surface morphology in the enzymatic hydrolysis of bacterial PHB.

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