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Heterogeneous kinetics of the enzymatic degradation of poly(β-hydroxyalkanoates)

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The kinetics of the enzymatic degradation reactions of the naturally produced polyesters poly(β-hydroxybutyrate) (PHB) and poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) (PHBV) by appropriate depolymerases were studied. Specifically, the interaction between the extracellular PHB depolymerase of *Pseudomonas lemoignei* and both PHB powder and solution-cast films of PHBV was investigated. Kinetic analysis of titristatically and turbidimetrically determined rates of degradation has revealed that the observed degradation behaviour was inconsistent with classical Michaelis–Menten enzymatic kinetics. Depending upon the relative concentrations of enzyme and substrate, each exhibited a range of kinetic dependencies from zero order to first order. At high relative concentrations of enzyme, the reaction rate became inversely proportional to enzyme concentration. A new kinetic treatment was derived which predicted the observed variations in kinetic dependence on both enzyme and substrate, and it successfully described previously published kinetic data for this and other PHB depolymerases. The degradation of PHB by the PHB depolymerase of *Aspergillus fumigatus* M2A was investigated for comparison. While the specific rate constants of this enzyme differed from that of *P. lemoignei*, the general kinetic model was still applicable.

(Keywords: polyhydroxyalkanoates; enzyme; biodegradation)

INTRODUCTION

Poly(β-hydroxybutyrate) (PHB) is a naturally occurring polyester produced by numerous bacteria as an osmotically inert means of storing carbon and energy. The bacteria that synthesize these polymers also produce the enzyme(s) needed for the subsequent intracellular depolymerization of the PHB to metabolizable, low molecular weight compounds. In addition to these bacteria, numerous other scavenger bacteria and fungi are capable of secreting extracellular PHB depolymerases (PHBases), which catalyse the hydrolysis of PHB and similar polymers, because these microorganisms are also capable of metabolizing the organic hydroxy-acids produced.

Several studies of the enzymatic degradation of PHB and related polymers have been published, but until recently there had been little effort to formulate even a crude mechanistic kinetic model of this reaction. Rather, many investigators have tried to fit their data to existing enzyme kinetics models, such as the Michaelis–Menten model. Such an approach has been used to determine the values of some kinetic constants, such as the Michaelis–Menten parameters v_{max} and K_m (as discussed below), for the depolymerases from several microorganisms, but often these values were obtained by studying the degradation of soluble oligomers. Nonetheless, K_m values ranging from 73 to 131 $\mu g\,ml^{-1}$ have been reported for the two PHBase isozymes from *Pseudomonas lemoignei*[1,2]. These values were determined from the degradation of insoluble, powdered PHB, presumably using isolated granules, although little detailed information was provided on the experimental conditions under which these constants were obtained.

Traditional Michaelis–Menten kinetics applied in these earlier studies were originally derived for homogeneous reaction systems in which both the enzyme and the substrate are soluble. Because PHB is an insoluble substrate, its enzymatic degradation is a heterogeneous reaction, and the kinetic models need to be altered to reflect this. The development of such a modified kinetics treatment was the primary objective of the research described in this paper. In order to generate kinetic data with which to test the various kinetic models that have been published, samples of PHB powder and poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) (PHBV) solvent-cast film were subjected to degradation by fraction A of the PHBase from *P. lemoignei* at several substrate and enzyme concentrations, and the reactions were monitored by titrimetry and turbidimetry. The kinetics models tested are described below.

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THEORETICAL TREATMENT OF DEGRADATION KINETICS

Homogeneous kinetics

Classical Michaelis–Menten enzyme kinetics were derived by assuming a series of elementary reactions between unbound substrate, S, unbound enzyme, E, and bound enzyme–substrate complex, ES. Under the assumption that enzyme is the limiting reactant, the rate of reaction can be written in the form shown in equation (1)[3–5]

$$v_0 = k_2[\mathrm{ES}] = \frac{k_2[\mathrm{E}]_0[\mathrm{S}]_0}{[\mathrm{S}]_0 + K_\mathrm{m}} \tag{1}$$

Inspection of equation (1) reveals that at all concentrations the reaction is predicted to be linear with respect to enzyme concentration, but that the substrate dependency will range from first order (when $[\mathrm{S}]_0 \ll K_\mathrm{m}$) to zero order (when $[\mathrm{S}]_0 \gg K_\mathrm{m}$). The maximum velocity for such a reaction will occur when the enzyme is completely in the bound form, $[\mathrm{E}]_0 = [\mathrm{ES}]$, as given in equation (2).

$$v_\mathrm{max} = k_2[\mathrm{E}]_0 \tag{2}$$

Incorporation of equation (2) into the reciprocal of equation (1) provides the Lineweaver–Burk linearization of the Michaelis–Menten kinetics equation:

$$1/v_0 = \frac{[\mathrm{S}]_0 + \mathrm{K_m}}{k_2[\mathrm{E}]_0[\mathrm{S}]_0} = \frac{1}{v_\mathrm{max}} + \frac{K_\mathrm{m}}{v_\mathrm{max}[\mathrm{S}]_0} \tag{3}$$

In the opposite extreme, where substrate is the limiting reactant, equations (1) through (3) take on different forms, where the $[\mathrm{S}]_0$ and $[\mathrm{E}]_0$ terms are interchanged[4,5]. Equations (4) to (6) represent substrate-limiting Michaelis–Menten kinetics.

$$v_0 = k_2[\mathrm{ES}] = \frac{k_2[\mathrm{E}]_0[S]_0}{[\mathrm{E}]_0 + K_\mathrm{m}} \tag{4}$$

$$v_\mathrm{max} = k_2[\mathrm{S}]_0 \tag{5}$$

$$1/v_0 = \frac{[\mathrm{E}]_0 + K_\mathrm{m}}{[\mathrm{S}]_0[\mathrm{E}]_0} = \frac{1}{v_\mathrm{max}} + \frac{K_\mathrm{m}}{v_\mathrm{max}[\mathrm{E}]_0} \tag{6}$$

No provision is made in either equation (1) or equation (4) for the case where $[\mathrm{E}]_0$ and $[\mathrm{S}]_0$ are of comparable magnitude.

Heterogeneous kinetics

The preceding kinetics treatments presume that both the enzyme and the substrate are soluble. However, many natural enzymatic reactions take place under conditions where the substrate is insoluble, as with PHB, such that the reaction is limited entirely to the surface of the substrate; hence, traditional homogeneous kinetics should not apply. Accordingly, McLaren[6] proposed a heterogeneous kinetics treatment in which it was explicitly recognized that only the surface of the insoluble substrate was accessible to the enzyme. McLaren further assumed that the adsorption of the enzyme onto the surface of the substrate obeyed a Freundlich isotherm of the form given in equation (7).

$$[\mathrm{ES}] = K[\mathrm{E}]^n \tag{7}$$

For adsorption of enzyme from a solution phase onto a two-dimensional insoluble surface, n is predicted to be 2/3. Thus, McLaren presumed that because the rate of the degradation reaction was directly proportional to [ES], it should be proportional to $[\mathrm{E}]^{2/3}$:

$$v_0 = k_2K[\mathrm{E}]^{2/3} \tag{8}$$

McLaren successfully applied such a two-thirds power-law to previously published data from numerous heterogeneous catalytic systems, including the enzymatic hydrolysis of cellulose, starch, protein gels, and oil emulsions, and demonstrated a power slope close to 2/3 for each case where enzyme was limiting[6].

More recently, Mukai and coworkers[7] proposed an alternate heterogeneous kinetics treatment specific to the enzymatic degradation of PHB. In contrast to both the traditional Michaelis–Menten and McLaren derivations, Mukai's treatment assumes a different interaction between the enzyme and the substrate. That is, consistent with experimental evidence which has shown that the enzyme contains discrete hydrophobic binding and catalytic domains[8], the enzyme is assumed first to bind to the polymer substrate and then subsequently to catalyse an hydrolytic scission at a different site on the substrate, as shown below in equation (9)

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \tag{9a}$$

$$\mathrm{ES} + \mathrm{S} \xrightarrow{k_2} \mathrm{ES} + \mathrm{P} \tag{9b}$$

Assuming the hydrolysis reaction (equation (9b)) to be the rate-limiting one, Mukai *et al.*[7] derived a rate equation of the form given in equation (10),

$$v_0 = \frac{k_2K[\mathrm{E}]_0}{(1 + K[\mathrm{E}]_0)^2} \tag{10}$$

where K is the adsorption equilibrium constant (k_1/k_{-1}). From equation (10), it can be seen that, at low enzyme concentrations ($K[\mathrm{E}]_0 \ll 1$), the reaction is predicted to be linear in enzyme concentration, as with Michaelis–Menten kinetics. Conversely, at a sufficiently high enzyme concentration ($K[\mathrm{E}]_0 \gg 1$), the reaction is predicted to be inversely proportional to the enzyme concentration. That is, beyond a certain 'optimal' concentration of enzyme ($K[\mathrm{E}]_0 = 1$), the reaction rate is predicted to decrease with an increase in $[\mathrm{E}]_0$. Mukai and coworkers[7] demonstrated this phenomenon for the degradation of PHB by employing a number of PHBases and were able to fit a linearized version of equation (10) to the data. However, although there is no explicit provision in equation (10) for a dependency of rate upon substrate concentration, they showed that such a dependency did exist.

EXPERIMENTAL

Polymer samples

The samples of biologically produced PHB and PHBV used in these studies were purchased from Polysciences and used without further purification. Stock suspensions of PHB powder in distilled water ranging in concentration from 125 to 1000 μg ml^{-1} were prepared by ultrasonic agitation. The PHBV contained 22 mol% hydroxyvalerate units, as verified by gas chromatography of the methanolyzed polymer[9]. Films of PHBV approximately 100 μm thick were cast from 5% (w/v) solutions in chloroform onto glass casting dishes. It was determined by scanning electron microscopy (SEM) that the PHB

particles used in these experiments were essentially smooth spheres with a very narrow size distribution centered around an average diameter of 600 nm[10]. For such particles, a specific surface area of $8.0\,m^2\,g^{-1}$ was computed, a result which was corroborated by measuring the surface area by nitrogen adsorption[11].

Depolymerase

The depolymerase obtained from cultures of *P. lemoignei* grown on PHB was purified by employing a combination of published protocols described elsewhere[12–14]. Depolymerase obtained from cultures of *Aspergillus fumigatus* grown on PHB was similarly isolated[15]. Purity of the enzyme preparations at various stages of the purification was determined by testing for enzymatic activity via the turbidimetric powder assay (see below) and polyacrylamide gel electrophoresis (PAGE). Fraction A of the *P. lemoignei* PHB depolymerase system[12] was used for the turbidimetry and titrimetry experiments.

Turbidimetric powder assay

The turbidimetric powder assay has been used in many studies to measure the rate of PHB degradation[1,2,13–17]. In this simple procedure, the decrease in light scattering caused by disappearance of the powdered substrate is measured. This decrease is a direct indicator of degradation, and has been correlated with the direct chemical technique of titrimetry (see below)[17]. This assay was used as a quick method to both measure and define enzymatic activity. Enzyme activity was determined using 0.67 ml of a 300 $\mu g\,ml^{-1}$ suspension of PHB powder finely dispersed by ultrasonication combined in a 1-cm pathlength plastic cuvette with 0.33 ml of enzyme solution (thus giving a final PHB concentration of $200\,\mu g\,ml^{-1}$); this mixture was then allowed to incubate at 30°C. As measured in a scanning u.v./vis spectrophotometer, such a suspension had an initial optical density at 660 nm (OD_{660}) of approximately 0.80. As the PHB was degraded by the enzyme, the optical density decreased, and enzymatic activity was defined in terms of the rate of change of OD_{660}. One unit (U) of enzyme activity was defined as that quantity which under these conditions would effect a change in OD_{660} of 0.001 (1 mOD) per minute.

For the purpose of the kinetic study, the assay was performed with a range of known enzyme and polymer concentrations, all in a total volume of 1.0 ml. The same lot of PHB was used for all experiments described herein, although it has been shown that different lots of PHB gave essentially identical degradation rates[17].

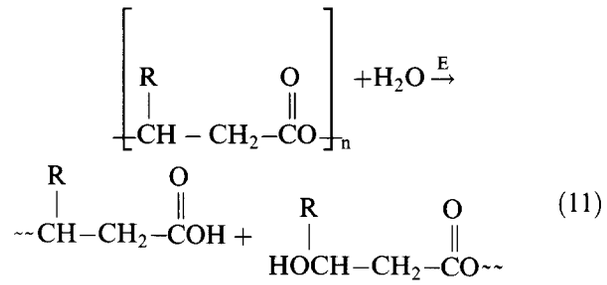
The titristatic method

This assay[17,18] was used to ascertain the initial rate of enzymatic degradation by continually titrating the organic acids produced as a result of the degradation reaction and measuring the rate at which sodium hydroxide had to be added to maintain pH 8.00. Into a 13 × 100 mm test tube partly immersed in a temperature-controlled water bath were placed a 10-mm magnetic stirbar, the sample of PHB or PHBV to be degraded, 2 or 4 ml of water, and, finally, an aliquot of purified PHB depolymerase. When PHBV film was used, the sample was weighed, its macroscopic dimensions measured (to determine the initial surface area), and introduced into the fluid, such that it was completely immersed. When PHB powder was used, an aliquot of a concentrated stock suspension of PHB was also used, with enough additional water added to bring the total initial volume to the desired value.

RESULTS AND DISCUSSION

PHB powder/PHBV film titrimetry

Titrimetric determinations of degradation rates are quantitative because they measure directly the degradation reaction by titrating the acid groups formed as a result of ester bond hydrolysis, as shown in equation (11), where R is CH_3 for PHB and CH_3 or CH_2CH_3 for PHBV:



(11)

This assay was applied both to various concentrations of PHB powder in suspension and to films of various sizes cut from one large film of solvent-cast PHBV. In all cases, the polymer was subjected to degradation using varying amounts of purified PHBase A from *P. lemoignei*.

Two similar sets of titration experiments were performed using PHB powder. In the first set, 2-ml reaction volumes were used, and reaction rates were measured for a series of four enzyme concentrations for the fixed surface concentration of PHB equal to

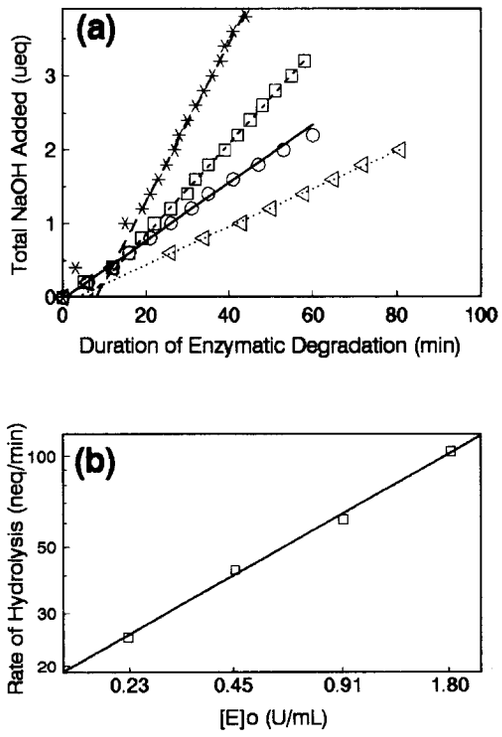


Figure 1 Titrimetric rate data for enzymatic degradation of 0.5 mg PHB powder in 2.0 ml reaction volumes. (a) Observed titrimetry data, with least squares fits to determine initial rate, for $[E]_0 = 1.81$ (*), 0.91 (□), 0.45 (○) and 0.23 (△) U ml^{-1}; (b) log–log plot of initial rates as a function of $[E]_0$

$20\,cm^2\,ml^{-1}$ (0.25 mg ml). The results of this set of experiments are shown in *Figure 1*. *Figure 1a* shows the titration curves used to determine the degradation rates. The double-log plot of rate vs $[E]_0$ shown in *Figure 1b* indicates that, indeed, the degradation rate increased as the concentration of enzyme was increased, but in a nonlinear fashion because a power-law slope of approximately 0.67 was observed. Thus, the reaction rate was not first-order in enzyme concentration, and the heterogeneous kinetics treatment of McLaren[6] (equation (8)), which predicts a 2/3 slope, appears to apply very well here.

Three additional sets of PHB powder degradation experiments were performed, for which 4 ml reaction volumes were used. These rate data are shown in *Figure 2*. Again, in each case an $[E]_0$ power dependence very close to 0.67 was obtained.

Because films of PHBV have a much lower surface-to-volume ratio than the powder, much higher $[E]_0/[S]_0$ ratios could be used. The rate data for the degradation of PHBV films are shown in *Figure 3*. From these data, the power dependence of the reaction rate as a function of enzyme concentration was calculated for each substrate concentration. This power-law slope is on the order of 0.30, which is much lower than the value of 0.67 obtained for the powder, but, once again, is not the value of unity which would have been predicted by a Michaelis–Menten treatment. Nonetheless, application of the Lineweaver–Burk linearization (equation (3)) afforded an excellent fit to the degradation rate data for each given enzyme concentration. Computed values of the standard kinetic parameters of K_m and v_{max}, along with the substrate power slopes discussed above, are listed in *Table 1*. It can be seen that a consistent value for the Michaelis–Menten constant of approximately $1.1\,cm^2\,ml^{-1}$ was obtained. Thus, while equation (1) would fail to predict a 0.30 or 0.67 power kinetic dependence on the concentration of the enzyme, for a fixed enzyme concentration it offered an excellent description of the substrate dependence.

In addition, it can be shown that the rate data in *Figure 3* are fitted by a 0.75 power dependence upon $[S]_0$, as listed in *Table 1*. According to equation (1), this result would mean that $[S]_0$ was neither much larger nor much smaller than K_m. Given the ascertained value for K_m of $1.1\,cm^2\,ml^{-1}$, it can be seen that, indeed, $[S]_0$ and K_m were of comparable magnitude.

Inadequacies of the Michaelis–Menten treatment notwithstanding, this $1.1\,cm^2\,ml^{-1}$ value for K_m agrees within an order of magnitude of those listed above[1,2], which averaged $0.1\,mg\,ml^{-1}$. If it is assumed (for lack of evidence to the contrary) that the values of K_m reported above were determined from degradation of isolated PHB granules similar to those used in the present study, then the values can be compared directly by conversion from mass concentration units to surface area concentration units. Assuming a PHB specific surface area[11] of $8.0\,m^2\,g^{-1}$, the $0.1\,mg\,ml^{-1}$ value converts to $8.0\,cm^2\,ml^{-1}$, which is within an order of magnitude of the $1.1\,cm^2\,ml^{-1}$ value reported here. If the PHB used in the earlier studies had been of a lower specific surface area than that used in the present studies, the two values would agree even more closely.

Curiously, it is equation (4) which correctly describes the kinetic dependence upon enzyme concentration of the PHB powder degradation rate as shown in *Figure 4*. Similar results have been reported for the degradation of PHB powder with this same enzyme[5,17]. However, as was mentioned above, the reaction rate in the present study was not linear in substrate concentration, as would be predicted by equation (4).

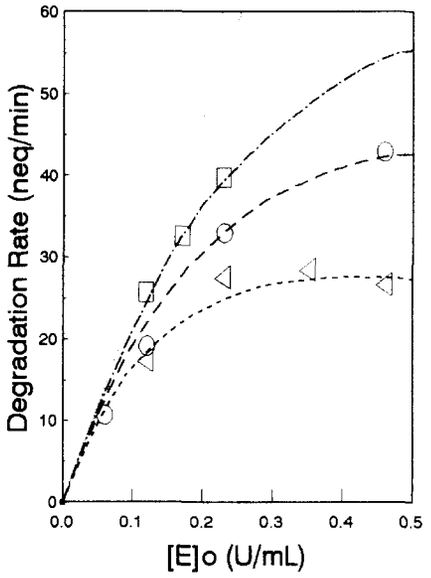


Figure 2 Titrimetric rate data for degradation of PHB powder plotted as a function of $[E]_0$ for three values of $[S]_0$: $21\,cm^2\,ml^{-1}$ (□) (250 μg ml^{-1}); $10\,cm^2\,ml^{-1}$ (○) (125 μg ml^{-1}); $5.0\,cm^2\,ml^{-1}$ (△) (63 μg ml^{-1}). Curves through the points represent the fit to each set of data of equation (16)

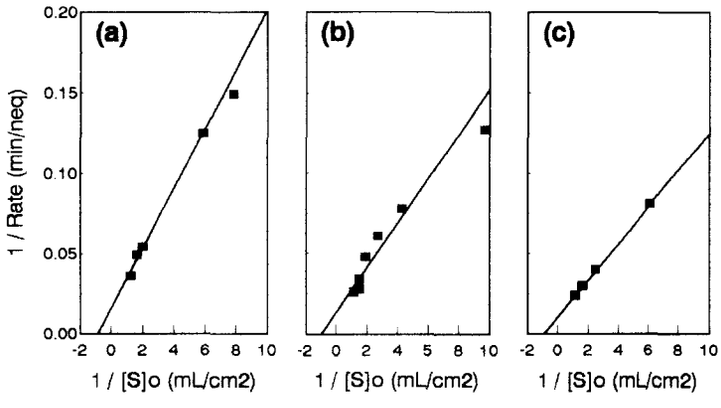


Figure 3 Traditional Lineweaver–Burk plots of equation (3) for PHBV film titrimetry rate data, for each of three values of $[E]_0$: (a) 3.6 nM (0.45 U ml^{-1}); (b) 7.3 nM (0.91 U ml^{-1}); (c) 14.5 nM (1.83 U ml^{-1})

Table 1 Michaelis–Menten kinetic parameters determined by fitting equation (3) to the data in *Figure 3*. Degradation of films of PHBV, approximately 50 μm thick, was conducted in 4 ml of enzyme solution containing varying amounts of *Pseudomonas lemoignei* PHBase

$[E]_0$ (nM)	$[S]_0$ Power-law slope	v_{max} (nEq min^{-1})	K_m (cm^2 ml^{-1})
1.8	0.75	60	1.1
3.6	0.75	72	1.0
7.3	0.75	94	1.1

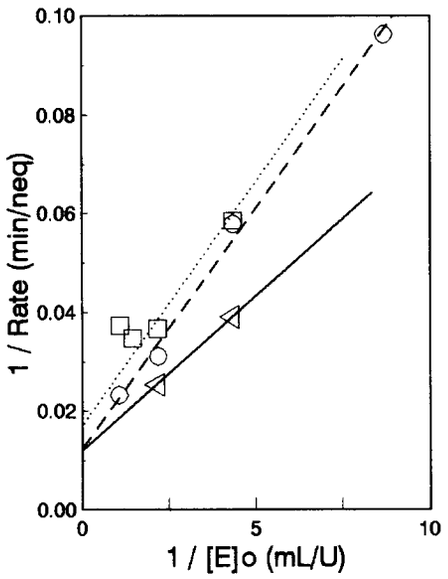


Figure 4 Substrate-limiting Lineweaver–Burk-type double reciprocal plots of equation (6) for PHB powder titrimetry rate data, for each of three values of $[S]_0$: 5.0 cm^2 ml^{-1} (□) (0.13 μg ml^{-1}); 10 cm^2 ml^{-1} (○) (0.25 μg ml^{-1}); 20 cm^2 ml^{-1} (△) (0.50 μg ml^{-1})

Thus, while it can be seen that equations (1), (4), and (8) all appear to apply to some extent under various limiting circumstances, inspection of the data in *Figures 1* through *4* reveals that none of these equations correctly predicted the degradation rate behaviour of both the powder and the film, because none of these equations correctly allows for simultaneous variations in the kinetic dependence of both enzyme and substrate. Because each of these kinetic equations was derived under a different set of limiting assumptions, each is only applicable—and then only partially—in the regime where its specific assumptions are valid.

The recent publication of Mukai and coworkers on the heterogeneous kinetics of PHB enzymatic degradation[7] has challenged the traditional paradigm concerning this reaction by introducing the notion that the substrate partakes twice in the degradation reaction, as outlined in equation (9). While the results presented above are consistent with this new kinetics treatment, the characteristic decrease in rate at large enzyme concentrations was not observed. In order to test more fully the general applicability of Mukai's kinetics, the range of concentrations explored had to be expanded. The results of such experiments are described in the following section.

PHB powder turbidimetry

Numerous experiments conducted at a variety of both enzyme and polymer concentrations were followed by means of powder turbidimetry, specifically for the purpose of studying the kinetics of the degradation reaction. The rate of the degradation reaction was defined as the initial slope of the plot of OD vs time. *Figure 5* shows the tubidimetrically determined degradation rates of PHB at a number of different values of $[S]_0$ in 1.0 ml total volume. This figure reveals that, as would be predicted by the treatment of Mukai and coworkers, at large relative enzyme concentrations the rate decreased with increasing $[E]_0$. Further conclusions about the kinetics drawn from these data are discussed below.

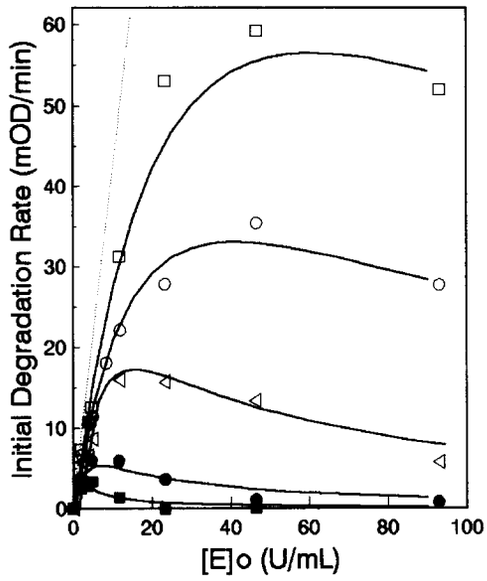


Figure 5 Turbidimetry rate data for enzymatic degradation of PHB powder in 1.0 ml reaction volumes, employing varying quantities of the PHBase from *Pseudomonas lemoignei*. Initial degradation rates are plotted as a function of $[E]_0$ (U ml^{-1}) for five values of $[S]_0$ in (cm^2 ml^{-1}): 36 (□); 24 (○); 12 (△); 4.8 (●); 2.4 (■). The curves through the points represent the fit to each set of data of equation (16) as shown in *Figure 6*

Modified heterogeneous kinetics

New heterogeneous kinetic model. The data in *Figure 5* reveal that, in addition to the kinetic dependence upon enzyme concentration predicted by the treatment given in equation (10), there was a strong dependence upon substrate concentration. The kinetic treatment of Mukai *et al.*[7] did not allow for such variation, although they reported that different rate constants were obtained when different substrate concentrations were employed. For every enzyme concentration, at small $[E]_0/[S]_0$ ratios the rate was essentially linear in enzyme and zero order in substrate, such that the initial portions of each experimental curve superimpose the dotted line given in *Figure 5*. Such behaviour would be predicted by traditional Michaelis–Menten (equation (1)) in such a concentration regime. At larger relative enzyme concentrations, the kinetic dependence upon substrate concentration was not zero-order, as shown by the deviation of each curve from the dotted line. Because they fail to account for such variation, the kinetic equations derived by Mukai and coworkers incompletely describe the kinetics of this reaction. Therefore, the derivation described below of a new heterogeneous kinetics treatment is specifically designed to account for the kinetic contributions of both enzyme and substrate concentrations.

Because the assumption that the substrate appears twice in the overall degradation reaction (i.e., once during the formation of the enzyme–substrate complex and once again during the hydrolysis reaction) is consistent with published data which indicate that many PHBases have discrete binding and catalytic domains[8], the derivation will commence from equation (9). Furthermore, it is assumed that the adsorption reaction obeys a Langmuir isotherm (demonstrated below), as given by equation (12).

$$d[ES]/dt = 0 = k_1[E][S] - k_{-1}[ES] \quad (12)$$

From equation (12), the concentration of the enzyme–substrate complex, [ES], is as given by equation (13),

$$[ES] = K_L[E][S] = K_L([E]_0 - [ES])([S]_0 - [ES]) \quad (13)$$

where K_L is the Langmuir adsorption equilibrium constant (k_1/k_{-1}). Equation (13) is quadratic in [ES], and is simplified to equation (14), assuming, for the sake of simplicity, that the $[ES]^2$ term is negligible (see Appendix):

$$[ES] = [E]_0[S]_0/(K + [E]_0 + [S]_0), \quad (14)$$

where $K = 1/K_L$. Therefore, assuming the hydrolysis reaction [i.e. equation (9b)] to be rate-limiting, the following rate equation can be derived:

$$v_0 = k_2[ES][S]/[S]_0 = \frac{k_2[E]_0[S]_0(K + [S]_0)}{(K + [S]_0 + [E]_0)^2} \quad (15)$$

Unlike equation (10), this equation explicitly allows for variations in substrate concentration.

The substrate concentration dependence of equation (9b) is given in the middle term in equation (15) by $[S]/[S]_0$. While formation of the enzyme–substrate complex is dependent upon the absolute concentrations, $[E]_0$ and $[S]_0$, the subsequent surface reaction depends only upon the fractional concentration of free substrate sites (i.e., $[S]/[S]_0$), and not on the absolute concentration of such sites. Because the two species which need to react are already in contact, their absolute concentrations in the reaction medium are irrelevant. Failure to include such a normalization results in a model which incorrectly predicts the substrate kinetic dependencies observed.

At low enzyme concentrations ($[E]_0 \ll K + [S]_0$), the reaction given by equation (15) should be linear in enzyme concentration, as it would be with Michaelis–Menten kinetics. In contrast, at sufficiently high enzyme concentrations ($[E]_0 \gg K + [S]_0$), v_0 should be inversely proportional to the enzyme concentration. That is, beyond a certain 'optimal' concentration of enzyme ($[E]_0^{opt} = K + [S]_0$, as shown below), the reaction rate is predicted to decrease with an increase in $[E]_0$. Both trends agree with the data observed in *Figure 5*.

The rate equation expressed in equation (15) can be linearized into the form shown in equation (16), which is similar to the linearization employed by Mukai *et al.*[7]

$$([E]_0/R)^{1/2} = \frac{K + [S]_0 + [E]_0}{\{k_2[S]_0(K + [S]_0)\}^{1/2}} = (\beta^2 + [E]_0)/\alpha\beta \quad (16)$$

where the following substitutions have been made:

$$\alpha = (k_2[S]_0)^{1/2} \quad (17a)$$

$$\beta = (K + [S]_0)^{1/2} \quad (17b)$$

The following expressions obtained from equation (17) allow for the kinetic constant k_2 and K to be evaluated.

$$k_2 = \alpha^2/[S]_0 \quad (18a)$$

$$K = \beta^2 - [S]_0 \quad (18b)$$

The experimental data from *Figure 5* are plotted in *Figure 6* as $([E]_0/R)^{1/2}$ vs $[E]_0$, and a least-squares fit of

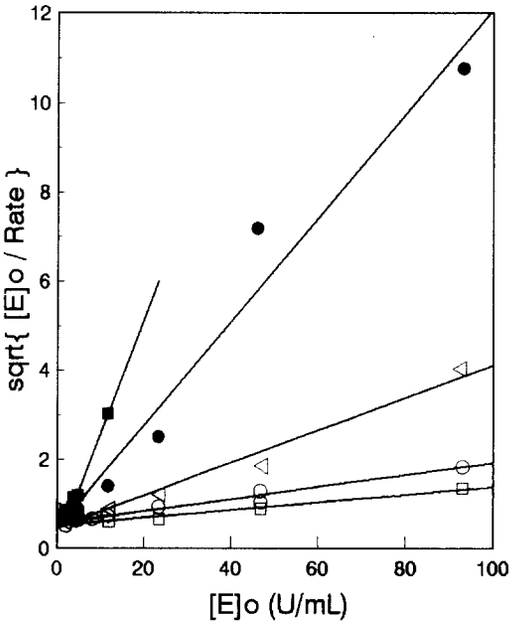


Figure 6 Linearized plot of the data in *Figure 5* to equation (16). $([E]_0/R)^{1/2}$ is plotted as a function of $[E]_0$ (U ml^{-1}) for five values of $[S]_0$ in (cm^2 ml^{-1}): 36 (□); 24 (○); 12 (△); 4.8 (●); 2.4 (■). The curves through the points represent least squares fits to each set of data of equation (16)

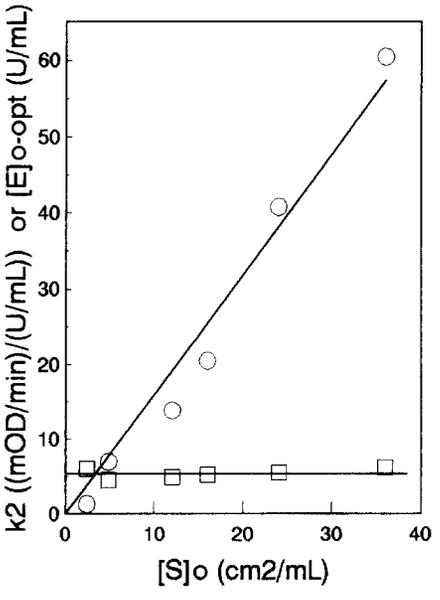


Figure 7 Values determined from the linear fits to the degradation rate data presented in *Figure 6*, as a function of $[S]_0$, of: k_2 (□) and $[E]_0^{opt}$ (○)

each set of data to equation (16) is used to determine the slope, $1/\alpha\beta$, and the intercept, β/α. The curves through the experimental points in *Figure 5* are based upon these least-squares fits.

Optimal enzyme adsorption density. By equating to zero the derivative with respect to $[E]_0$ of equation (15), the optimal value of $[E]_0$ (i.e., that which gives the maximum rate for a given $[S]_0$) is obtained as: $[E]_0^{opt} = K + [S]_0 = \beta^2$. This value corresponds to the inverse of the x-axis intercept of equation (16). The maximum rate itself is given by $\frac{1}{4}k_2[S]_0$. The enzyme turnover number, k_{cat}, defined as the maximal rate divided by $[E]_0^{opt}$, can be evaluated as $k_{cat} = (\frac{1}{4}k_2[S]_0)/(K + [S]_0)$, and this value reduces to $k_{cat} = \frac{1}{4}k_2$ when $[S]_0 \gg K$.

Figure 6 reveals that equation (16) provides an excellent fit to the experimental data. The values for $[E]_0^{opt}$ and k_2 determined from the slopes and intercepts of each line in *Figure 6* are plotted in *Figure 7* against the values of $[S]_0$ corresponding to each set of experimental data. As shown in *Figure 7*, a constant value for the rate constant k_2 is obtained, contrary to what was reported by Mukai *et al.*[7]. In addition, the maximum reaction rate, v_{max}, is a linear function of the total substrate concentration, a finding which is both intuitive and consistent with claims made for this same enzyme by Delafield[16]. Moreover, the slope of the plot of v_{max} *vs* $[S]_0$ given in *Figure 7* corresponds to the optimal packing of enzyme per square centimetre of substrate, a parameter which is characteristic of a given preparation of enzyme. For the enzyme used in this set of experiments, optimal packing corresponded to approximately $1.5\,U\,cm^{-2}$.

Maximal enzyme adsorption density. In solving the kinetic constants, the units of measure for K, $[E]_0$, and $[S]_0$ had to agree, of course. That is, it was necessary to determine a conversion factor from cm^2 to U. The value of $\sim 1.5\,U\,cm^{-2}$ mentioned above represents only the optimal packing density of enzyme. What was needed was the actual surface area occupied per unit of enzyme activity; experiments performed to ascertain this conversion factor are described below.

In order to determine the conversion factor from U to cm^2, and also to demonstrate the validity of this kinetic model, the following experiment was performed. The rates of degradation of a small concentration of PHB ($[S]_0 = 60\,\mu g\,ml^{-1} = 4.8\,cm^2\,ml^{-1}$) with varying concentrations of enzyme (from 0 to $36\,U\,ml^{-1}$) were determined. The turbidimetric degradation rates obtained from this set of experiments are given in *Figure 8*. A parallel binding study was performed to ascertain what fraction of the initially added enzyme had adsorbed to the polymer. To make this measurement, experiments identical to those mentioned above were performed, except that after 5 min the reaction mixtures were centrifuged. The supernate was then assayed for residual activity via the standard turbidimetric assay. The value of [E], which is the concentration of unbound enzyme, was determined by this procedure, and because $[E]_0$ was known, it was possible to solve for [ES] by difference.

Determination of the values of [S] from $[S]_0 - [ES]$, however, required the aforementioned conversion factor. It was possible to determine the surface area of the substrate in terms of U, under the assumption that maximal adsorption represented complete coverage of the surface (i.e., that $[ES]_{max} = [S]_0$). Plots of [ES] against $[E]_0$ were fitted by a Langmuir isotherm, as shown in *Figure 9*, from which it could be determined

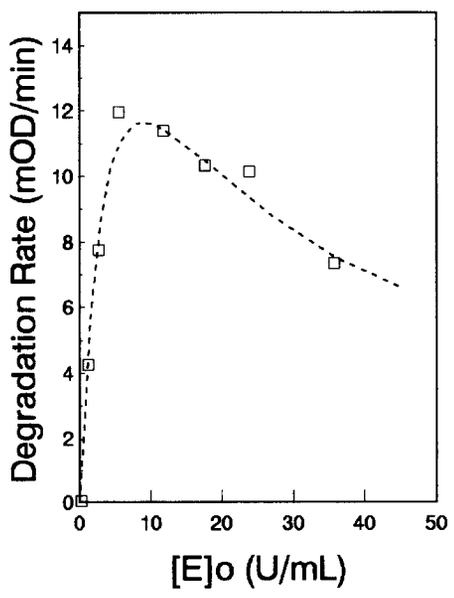


Figure 8 Turbidimetrically determined degradation rates of $4.8\,cm^2\,ml^{-1}$ PHB powder, as a function of $[E]_0$

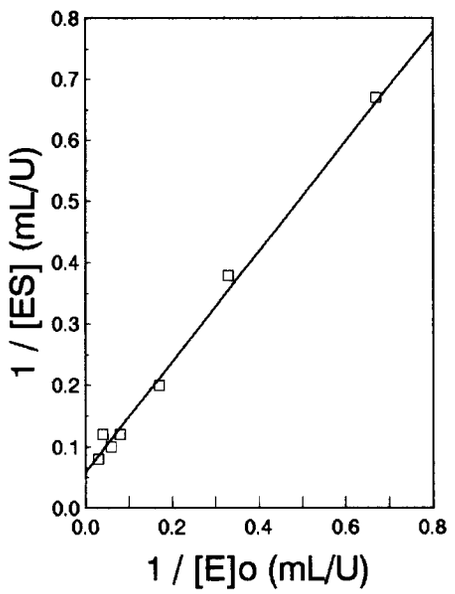


Figure 9 Fit of experimentally determined values of [ES] to a

Table 2 Binding equilibrium constants, $K = [ES]/([E][S])$, and fraction of total enzyme adsorbed, $[ES]/[E]_0$, from the data in *Figure 10*

$[E]_0$ ($U\,ml^{-1}$)	[E] ($U\,ml^{-1}$)	[S] ($U\,ml^{-1}$)	[ES] ($U\,ml^{-1}$)	K ($U\,ml^{-1}$)	$[ES]/[E]_0$ (%)
0.0	0.00	14.8	0.00		
1.5	0.00	13.2	1.50	∞	100
3.0	0.23	12.0	2.77	1.01	92
6.0	1.22	10.0	4.78	0.39	80
12.0	4.49	7.24	7.51	0.23	40
18.0	8.73	5.48	9.3	0.19	42
24.0	13.5	4.24	10.5	0.18	39
36.0	23.9	2.63	12.1	0.19	34

that $[ES]_{max}$, and therefore $[S]_0$, was equal to 14.9 U ml^{-1}. Thus, the conversion factor was 3.1 U cm^{-2} (i.e., 14.9 U ml^{-1}/4.8 cm^2 ml^{-1}).

From the results of this experiment, the value of the adsorption equilibrium constant, $K = [ES]/([E][S])$, and the fraction of adsorbed enzyme, $[ES]/[E]_0$ was computed; the quantities are listed in *Table 2*. It was found that K was approximately constant at a value of 0.2 at or above $[E]_0^{opt}$ for the fixed value of $[S]_0$ of 4.8 cm ml^{-1} $\approx$ 14.9 U ml^{-1}. It can be assumed that similar trends would be exhibited for other substrate concentrations, although it is likely that the actual, essentially constant value of K would vary with $[S]_0$.

Figure 10 shows a plot of [ES], [S], and the product of [ES] and [S] against $[E]_0$; the [ES] × [S] curve very closely resembles the original rate curve given in *Figure 8*, exhibiting a maximum and subsequent downturn at lower values of $[E]_0$ than the value of $[E]_0$ at which [ES] begins to plateau. The fact that the decrease in rate at large values of $[E]_0$ was not caused by parallel decrease in [ES] validates the assumptions made in equation (15) that the reaction rate is proportional to the product of [S] and [ES], and not simply [ES], as is the assumption in classical Michaelis–Menten kinetics (see equation (1)).

The results of the above experiment are consistent with the intuitive, conceptual explanation offered by Mukai *et al.*[7] for the decrease in rate at large $[E]_0$. Because the adsorption process is distinct from, and more rapid than, the hydrolysis reaction, it is possible to adsorb so much enzyme onto the surface that it begins to block itself. Thus, by serving to reduce the number of vacant substrate sites ([S]), it is proposed that large concentrations of adsorbed enzyme ([ES]) actually prevented access of the enzyme's catalytic domain to sites on the substrate surface. (Once again, traditional enzyme kinetics fail us, because, strictly speaking, the adsorbed enzyme inhibits not itself but rather the substrate, since it is with the substrate that the enzyme 'reacts'. However, traditional enzyme kinetic analysis[3,4] makes no provision for what is essentially competitive substrate inhibition.)

As can be seen in *Figure 10*, the optimal adsorption density of enzyme corresponded to the case where [ES] and [S] were equal, or when both of these concentrations equalled $\frac{1}{2}[S]_0$, since at this condition the product of [S] and [ES] was a maximum. Thus, for these experiments, $[S]_0$ = 14.9 U ml^{-1}, and $[E]_0^{opt}$ = 7.5 U ml^{-1}, as shown in *Figure 8*. Consistent with theory, the optimal adsorption density was half the maximal adsorption density.

Versatility of the heterogeneous kinetic model. The new kinetic model, given in equation (15), is both more accurate and more versatile than that given in equation (10). Not only does it address the decrease in rate at large enzyme concentrations, it also accounts for the kinetic

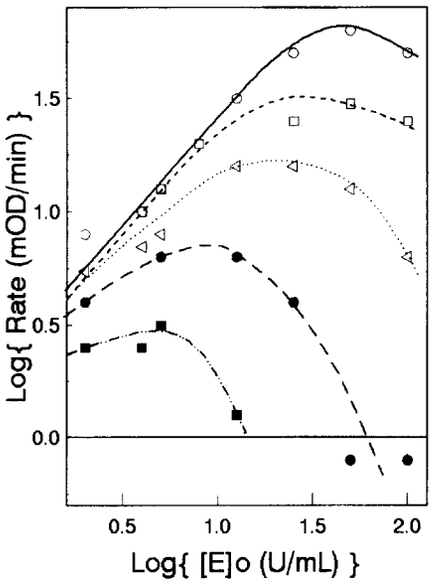


Figure 11 Double-log plot of the turbidemetry rate data from *Figure 5* for enzymatic degradation of PHB powder in 1.0 ml reaction volumes. Symbols are as defined in *Figure 5*

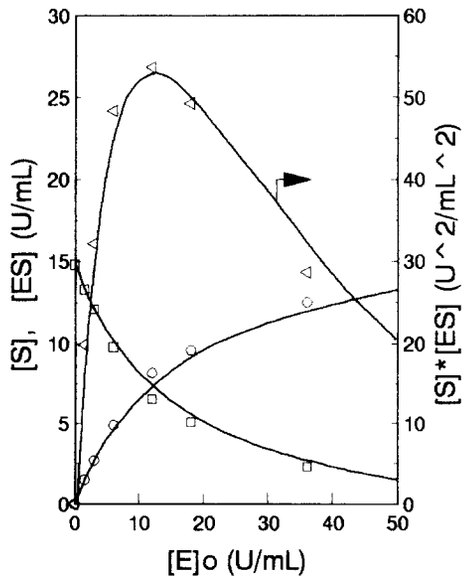


Figure 10 Values of [ES] (○) and [S] (□), experimentally determined by difference, as well as [ES] × [S] (△), plotted as a function of $[E]_0$. The points (○, □, and △) derive from the measurements, while the lines derive from the Langmuir fit of [ES] given in *Figure 9*

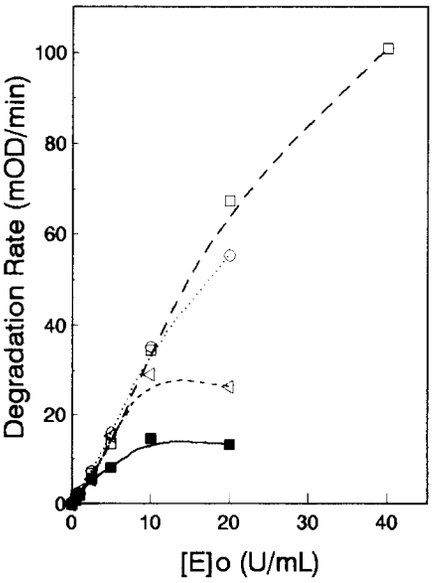


Figure 12 Initial rates of degradation at 45°C of PHB powder using the PHBase from *Aspergillus fumigatus*, as a function of $[E]_0$ (U ml^{-1}) for four values of $[S]_0$ (cm^2 ml^{-1}): (□) 24; (○) 12; (△) 6; (■) 2.4. Curves through the points represent the fit to each set of data of equation (16)

dependence upon both enzyme and substrate. As equation (15) would predict, the experimental data show that the rate varied between zero- and first-order in $[S]_0$. For low values of $[E]_0$ and large (relative) values of $[S]_0$, the reaction was zero order in substrate concentration (many of the curves in *Figure 5* were coincident) and linear in $[E]_0$. At larger values of $[E]_0$, the rate curves began to separate from each other, depending on the value of $[S]_0$. As is more obvious in the log–log plot in *Figure 11*, there was a sizeable regime with concentrations similar to those used in the PHB titrimetry experiments described above (i.e. $[S]_0 = 10\,cm^2\,ml^{-1}$ and suboptimal values of $[E]_0$) where the rate exhibited close to a 0.67 power dependence upon $[E]_0$. Because it has now been shown that the PHBase obeys a Langmuir-type isotherm (*Figure 9*), the results plotted in *Figure 11* explain why such a slope was observed in the titrimetry experiments. That is, the two-thirds power slope was not due to a Freundlich isotherm, as suggested by McLaren[6], but was merely a coincidence of the ($[E]_0$, $[S]_0$) concentration coordinates at which the measurements were made.

Because equation (15) is similar in form to equation (10), it also predicts the behaviour of the three PHBases investigated by Mukai and coworkers[7]. In addition, rate data obtained from turbidmetric degradation experiments performed at 45°C employing the PHBase from *A. fumigatus* M2A[15] were also fitted quite well by equation (15), as shown in *Figure 12*, although none of these plots demonstrates the eventual decrease in rate at high $[E]_0$, since only small concentrations of enzyme were used. Note that at low $[E]_0/[S]_0$ ratios the zero-order dependence upon substrate and the linear dependence upon enzyme predicted by equation (15) are very pronounced for this set of curves. In addition, equation (15) also fits the PHB powder titrimetry data discussed above, and the curves shown in *Figure 2* are from such fits.

Because the location of the maximum rate is a function of the concentrations of both the total protein in the system and the total active protein, the enzyme concentration which gives the maximum rate, $[E]_0^{opt}$, and, indeed, the maximum rate itself, will no doubt vary with the specific activity of the enzyme preparation used in a given set of experiments. Indeed, it can be seen that while *Figure 9* exhibits the same trend and the same $[E]_0^{opt}$ as the plot for $4.8\,U\,ml^{-1}$ in *Figure 5*, a higher v_{max} resulted, since a preparation of enzyme with a greater specific activity (30 vs $9\,U\,\mu g^{-1}$) was used for the binding study (*Figure 9*).

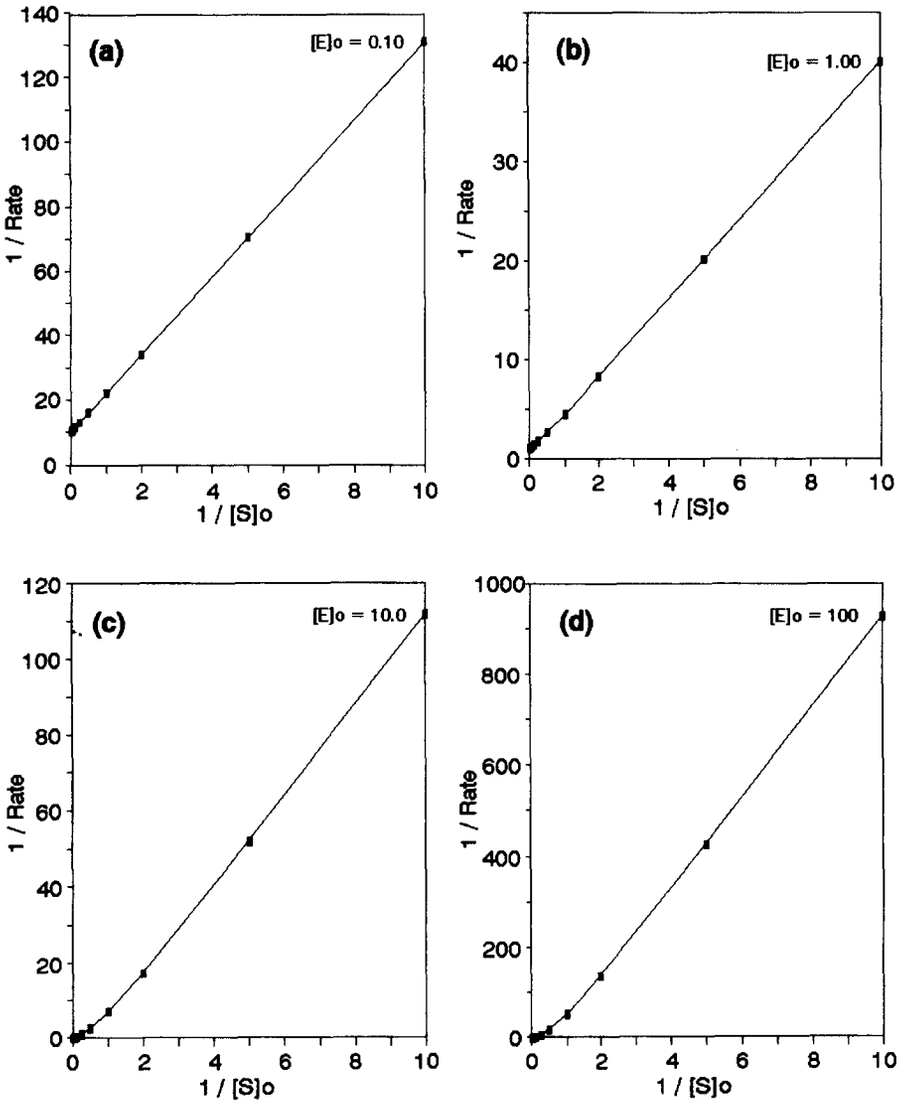


Figure 13 Double reciprocal, traditional Michealis–Menten-type plot of equation (15), with $k_2 = K = 1$. The reciprocal of the rate is plotted as a function of the reciprocal of $[S]_0$, for several $[E]_0$: (a) 0.10; (b) 1.0; (c) 10; (d) 100

Comparison of models

As discussed above, attempts to analyse the titrimetric rate data by means of traditional Michaelis–Menton type graphical analysis was only partially successful. It is now understood that this is because the mechanism presumed by such kinetics is incorrect. The heterogeneous kinetics treatment derived above, however, may also be applied graphically, in a fashion similar to the traditional Lineweaver–Burk double-reciprocal graphical method used to analyse Michaelis–Menten data. Equation (19) provides such a manipulation of equation (15).

$$1/v_0 = \frac{(K + [E]_0 + [S]_0)^2}{k_2[E]_0[S]_0(K + [S]_0)} \tag{19}$$

For very small values of $[E]_0$ (i.e., $[E]_0 \ll K + [S]_0 = E]_0^{opt}$), equation (4) reduces to the form shown in equation (20)

$$1/v_0 = \frac{K + [S]_0}{k_2[E]_0[S]_0} \tag{20}$$

which is directly analogous to the corresponding traditional enzyme-limiting equation (compare with equation (3)). At small values of $[E]_0$, the substrate is present in excess, and the reaction approaches zero-order in substrate, as shown by the dotted line in *Figure 5*. Thus, in this concentration regime there is little kinetic importance to the fact that the catalytically active domain and the adsorption domain of the enzyme must compete for vacant sites on the substrate surface.

Figure 13 shows hypothetical plots of equation (19), where for tutorial simplicity the values of K and k_2 have both been taken to be unity. Provided that the assumption used in deriving equation (20) is satisfied, namely $[E]_0 \ll K + [S]_0$, the curves in *Figure 13* resemble traditional Lineweaver–Burk plots, and the behaviour is as would be predicted by classical Michaelis–Menten kinetics. At higher values of $[E]_0$, however, negative Y-intercepts result from extrapolation of the linear region, which is meaningless from a Michaelis–Menten perspective.

Conversely, *Figure 14* shows several hypothetical, substrate-limiting-type double reciprocal plots of equation (19), where again the values of K and k_2 have both been taken to be unity. It can be seen that where $[S]_0$ is

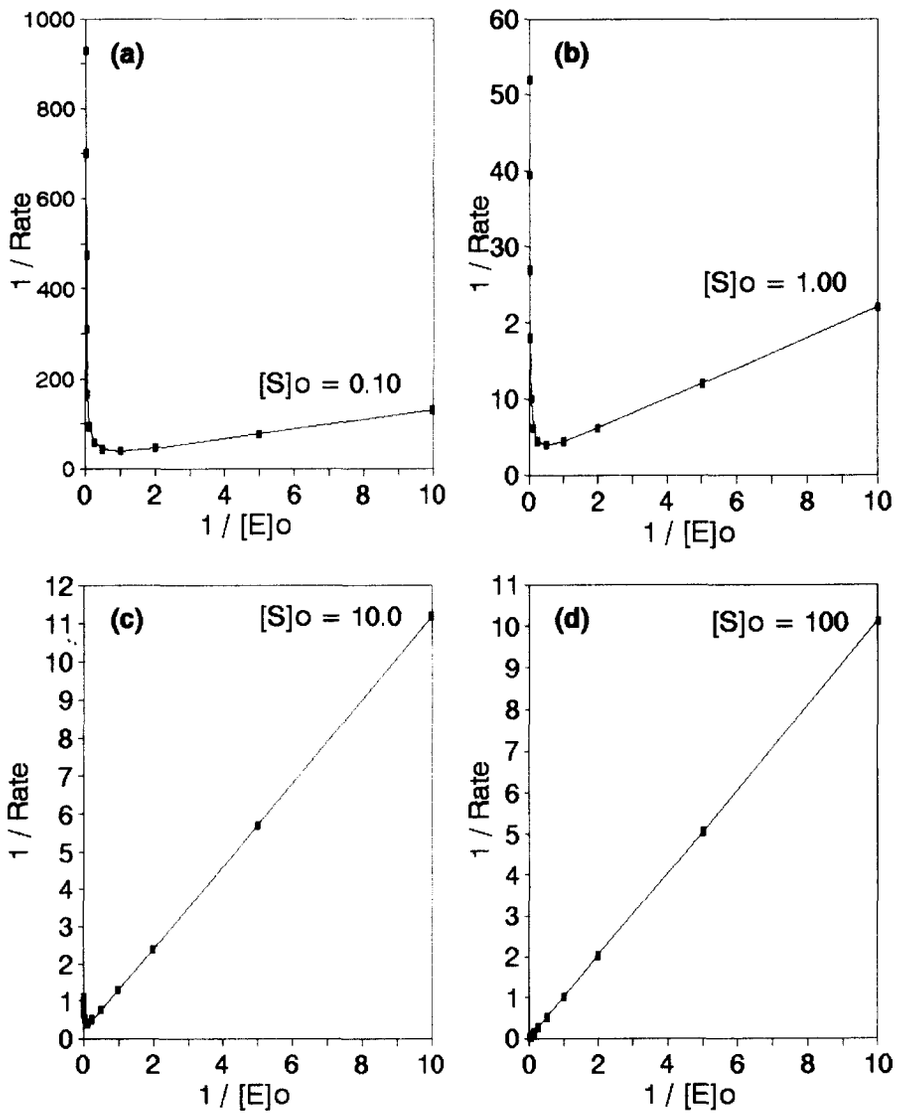


Figure 14 Double reciprocal, enzyme-limiting Michaelis–Menten-type plot of equation (15), with $k_2 = K = 1$. The reciprocal of the rate is plotted as a function of the reciprocal of $[E]_0$, for several $[S]_0$: (a) 0.10; (b) 1.0; (c) 10; (d) 100

much greater than $K + [E]_0$, the plots are linear. At very large values of $[S]_0$ (i.e., $[S]_0 \gg K + [E]_0$), equation (26) reduces to the form given in equation (21).

$$1/v_0 = \frac{1}{k_2[E]_0} \tag{21}$$

When the relative enzyme concentration approaches and exceeds $[E]_0^{opt}$, however, the curves deviate sharply from linearity; i.e., the behaviour ceases to resemble substrate-limiting Michaelis–Menten.

Hydrolysis rate constants

A plot of experimental data in the regime where $[S]_0 \gg K + [E]_0$ should yield a straight line with a slope equal to the reciprocal of the hydrolysis rate constant. *Figure 15* shows a plot in the style of *Figure 14* of the experimental rate data given in *Figure 5*. These plots are essentially identical to those predicted by equation (20) and *Figure 14*. For substrate concentrations greater than $24\,cm^2\,ml^{-1}$, the data superimpose each other in this plot because the substrates were in very large excess. In addition, as predicted by equation (21), the slope of the linear portion of these curves is equal to $1/k_2$. In agreement with the results shown in *Figure 7*, for these experimental data, $k_2 \approx 5\,mOD\,min^{-1}\,ml^{-1}\,U^{-1}$. Thus, simple graphical treatment of the data allows for determination of a fundamental kinetic constant.

Comparison of hydrolysis rate constants

Closer inspection of *Figure 5* reveals that the k_2 rate constant can also be determined from this plot by taking the slope of a tangent drawn through points where the rate curves superimpose (i.e., along the dotted line) because in this regime $v_0 = k_2[E]_0$. Determination of k_2 in this manner for the degradation rate data obtained using the *A. fumigatus* PHBase, as shown in *Figure 12*, yields a similar value: $3.5\,mOD\,min^{-1}\,ml^{-1}\,U^{-1}$. That a slightly smaller value was determined for reactions run at a temperature 15°C greater than that employed with the *P. lemoignei* enzyme implies that the fundamental rate constant for the fungal enzyme is smaller than that of *P. lemoignei*, particularly since it has been observed that this enzyme is nearly an order of magnitude more active at 45°C than at 30°C[14].

Because the experiments employing the *A. fumigatus* enzyme were performed at a higher temperature than those employing the *P. lemoignei* enzyme, it is probable that the two enzymes had different adsorption equilibria. Specifically, it is probable at 45°C that less of the *A. fumigatus* enzyme was adsorbed, since Delafield showed that the Langmuir adsorption of PHBase was inversely proportional to temperature[16]. This effect would explain the trend seen in *Figure 12* that over a large range of enzyme concentrations the reaction was zero order in substrate and linear in enzyme, since much more enzyme would have to be added to the system before half the substrate surface was covered.

The value of $5\,mOD\,min^{-1}\,ml^{-1}\,U^{-1}$ for the hydrolysis rate constant of the *P. lemoignei* enzyme can be converted to more meaningful units through knowledge of the concentration of the enzyme preparations and the polymer suspensions. The turbidity of suspensions of undegraded PHB were linear with PHB concentration up to an OD_{660} of 1.2, as shown in *Figure 16*. The slope of the plot in *Figure 16* allows conversion of turbidity units to mass concentration units, under the assumption that the correlation between turbidity and concentration was unaffected by the degradation of the particles. Because only initial degradation rates were used in the kinetics treatment, this assumption is probably valid. Because the enzyme used in the kinetic studies which yielded *Figure 5* had a concentration of $186\,U\,ml^{-1}$ and $20\,\mu g\,ml^{-1}$ (i.e. $9.3\,U\,\mu g^{-1}$), and a molecular mass of 55 kDa, the aforementioned value of k_2 can be converted to $10\,\mu g_p\,min^{-1}\,\mu g_e^{-1}$, where μg_p is polymer weight loss (in μg) and μg_e is the mass of enzyme present (in μg).

From the data in *Figure 2*, a value for the hydrolysis rate constant k_2 of $220\,nEq\,min^{-1}\,ml\,U^{-1}$ can be determined from a tangent to the curves. Since the enzyme preparation used in these studies was such that $0.61\,U\,ml^{-1}$ corresponded to 10 nM, and since one nEq

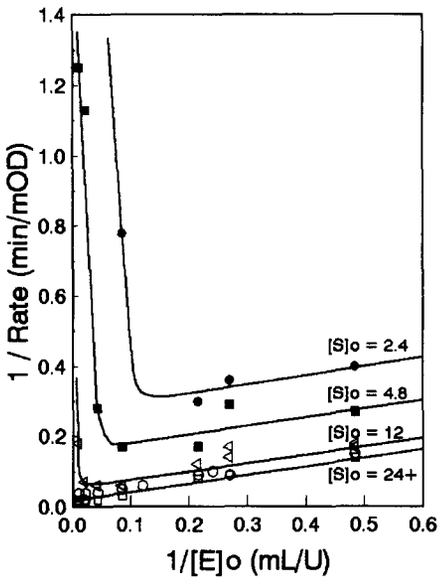


Figure 15 Double reciprocal, enzyme-limiting Michaelis–Menten type plot of the degradation rate data from *Figure 5*. The reciprocal of the rate is plotted as a function of the reciprocal of $[E]_0$ ($U\,ml^{-1}$), for several values of $[S]_0$ in ($cm^2\,ml^{-1}$): 36 (□); 24 (○); 12 (△); 4.8 (●); 2.4 (■)

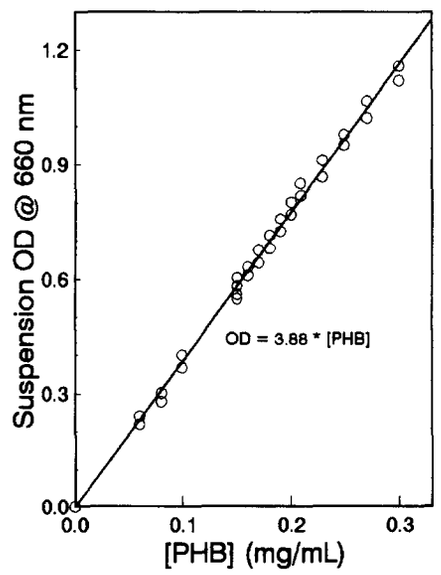


Figure 16 Correlation between turbidity and mass concentration of the stock suspensions of PHB used in the turdimetry experiments

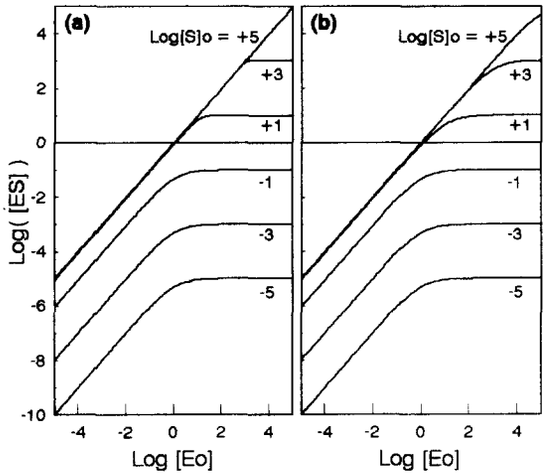


Figure A1 Values of [ES] computed from (a) equation (24) and (b) equation (25). The similarity of the two curves validates the assumption made in deriving equation (15) that $[ES]^2$ is negligible

of degraded PHB corresponds to 84 ng, this value for k_2 is identical to the value given above: $10\,\mu g_p\,min^{-1}\,\mu g_e^{-1}$. That the same value for k_2 could be determined from two separate sets of experiments argues strongly that it is the true value of this reaction rate constant.

CONCLUSIONS

A versatile new heterogeneous kinetics model specific to the enzymatic degradation of PHB/V has been developed. This new model describes observed degradation rate data both more thoroughly and more accurately than any kinetic models previously employed. Specifically, the new heterogeneous kinetics model predicts the degradation rate of PHB/V degradation over a large range of both enzyme and polymer concentrations. Neither Michaelis–Menten kinetics nor heterogeneous models developed by Mukai and McLaren[6,7] describe the full range of rate behaviour observed. Simple graphical methods based upon the new kinetics model were used to determine fundamental rate constants for the PHBase from the bacteria *P. lemoignei*. Analysis of two separate sets of degradation rate data generated with different preparations of this enzyme both exhibited the same rate constant: $10\,\mu g_p\,min^{-1}\,\mu g_e^{-1}$. The PHBase of the fungus *A. fumigatus* was also investigated; its hydrolysis rate constant appears to be smaller than that for the bacterial enzyme.

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REFERENCES

1 Nakayama, K., Saito, T., Fukui, T., Shirakura, Y. and Tomita, K. *Biochem. Biophys. Acta* 1985, **827**, 63
2 Mukai, K., Yamada, K. and Doi, Y. *Int. J. Biol. Macromol.* 1992, **14**, 235
3 Voet, D. and Voet, J. G. 'Biochemistry', John Wiley & Sons, New York, 1990.
4 Bailey, J. E. and Ollis, D. F. 'Biochemical Engineering Fundamentals', McGraw-Hill, New York, 1986
5 MacLaren, A. D. and Packer, L. 'Advances in Enzymology, Vol. 33' (Ed. F. F. Nord), Interscience, New York, 1970, p. 245
6 McLaren, A. D. *Enzymologia* 1963, **26**, 237
7 Mukai, K., Yamada, K. and Doi, Y. *Int. J. Biol. Macromol.* 1993, **15**, 361
8 Fukui, T., Narikawa, T., Miwa, K., Shirakura, Y., Saito, T. and Tomita, K. *Biochem. Biophys. Acta* 1988, **952**, 164
9 Lenz, R. W., Kim, Y. B. and Fuller, R. C. *J. Bioactive Compat. Polym.* 1991, **6**, 382
10 Timmins, M. R., Throckmorton, J. L., Garcia-Rubio, L. H., Fuller, R. C. and Lenz, R. W. in press
11 Timmins, M. R., Pullen, A., Conner, W. C., Fuller, R. C. and Lenz, R. W. in press
12 Lusty, C. J. and Doudoroff, M. *Proc. Natl Acad. Sci. USA* 1966, **56**, 960
13 Gilmore, D. F., Fuller, R. C. and Lenz, R. W. 'Degradable Materials: Perspectives, Issues, and Opportunities' (Eds S. A. Barenberg, J. L. Brash, R. Narayan, and A. E. Redpath), CRC Press, Boca Raton, FL, 1990, p. 481
14 Timmins, M. R., Gilmore, D. F., Fuller, R. C. and Lenz, R. W. 'Biodegradable Polymers and Packaging' (Eds D. Kaplan, E. Thomas, and E. Ching), Technomic, Lancaster, PA, 1993, 119
15 Scherer, T. M., Ph. D. Thesis, University of Massachusetts, Amherst, 1996
16 Delafield, F. P. Ph.D. Thesis, University of California, Berkeley, 1964
17 Hocking, P. J., Marchessault, R. H., Timmins, M. R., Fuller, R. C. and Lenz, R. W. *Macromolecules* 1996, **29**, 2472
18 Scherer, T., Fuller, R. C. and Lenz, R. W. *J. Environ. Polym. Degradation* 1994, **2**, 263

APPENDIX

It is required to prove the validity of the assumption used in deriving equation (15) that the $[ES]^2$ term is negligible. Start with equation (13):

$$[ES] = K_L([E]_0 - [ES])([S]_0 - [ES]) \qquad (13)$$

If, for simplicity, we let $K = 1/K_L$, $S = [S]$, $S_0 = [S]_0$, $E = [E]$, $E_0 = [E]_0$, and $Y = [ES]$, then equation (13) rearranges to yield equation (A1).

$$KY = (E_0 - Y)(S_0 - Y) \qquad (A1)$$

This is a quadratic equation in Y, which rearranges into the form shown in equation (A2).

$$Y^2 - Y(S_0 + E_0 + K) + E_0S_0 = 0 \qquad (A2)$$

Assuming that the Y^2 term is negligible allows for direct solution for Y, as given in equation (A3).

$$Y = E_0S_0/(E_0 + S_0 + K) \qquad (A3)$$

Using the quadratic formula to solve equation (A2) gives equation (A4).

$$Y = 0.5((S_0 + E_0 + K) \pm \{(S_0 + E_0 + K)^2 - 4(E_0S_0)\}^{1/2}) \qquad (A4)$$

Side-by-side comparison of the predicted values for Y for given values of E_0 and S_0 are shown in *Figure A1* for both equations (A3) and (A4), where it has been assumed, for simplicity, that K is unity. Note that only the 'plus' and not the 'minus' term in equation (A4) predicts realistic curves, and that these curves are almost identical to those predicted by equation (A3). The only minor differences in the curves occurs in the 'bend' region, at the onset of the plateau, where $E_0 \approx S_0$. Thus, assuming that the Y^2 term is negligible is a reasonable simplification.