ENZYMATIC DEGRADATION OF POLY-[(R)-3-HYDROXYBUTYRATE]: MECHANISM, KINETICS, CONSEQUENCES

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ABSTRACT

Poly-[(R)-3-hydroxybutyrate] (PHB) films prepared by compression molding and solvent casting, respectively, were degraded with the intracellular depolymerase enzyme natively synthetized by the strain *Bacillus megaterium.* Quantitative analysis proved that practically only (R)-3-hydroxybutyric acid (3-HBA) forms in the enzyme catalyzed reaction, the amount of other metabolites or side products is negligible. The purity of the product was verified by several methods (UV-VIS spectroscopy, liquid chromatography, mass spectroscopy). Degradation was followed as a function of time to determine the rate of enzymatic degradation. Based on the Michaelis-Menten equation a completely new kinetic model has been derived which takes into consideration the heterogeneous nature of the enzymatic reaction. Degradation proceeds in two steps, the adsorption of the enzyme onto the surface of the PHB film and the subsequent degradation reaction. The rate of both steps depend on the preparation method of the samples, degradation proceed almost twice as fast in compression molded films than in compression molded samples. The model can describe and predict the formation of the reaction product as a function of time. The approach can be used even for the commercial production of 3-HBA, the chemical synthesis of which is complicated and expensive.

1. INTRODUCTION

The threat of depleting fossil fuel sources and the increasing environmental awareness of the public generated growing interest in polymers produced from renewable resources [1-4]. However, the production of synthetic polymers still exceeds by far the amount of bioplastics produced and used thus also the quantity of plastics waste increases rapidly. Accordingly, besides bioplastics, biodegradable polymers gain more and more interest and importance as well. Presently the biodegradable polymer available in the largest

quantity on the market is poly(lactic acid), but it is produced by traditional chemical synthesis [4-10]. However, in the past few decades, several fermentation techniques were developed in order to facilitate the production of microbial polyesters [11-17] and reduce the price of the product. One of the most important and most frequently studied microbial polyesters is poly-[(R)-3-hydroxybutyrate]. [18-24].

The biodegradation of polymers is extremely important in several fields of application. The composting of packaging materials of short service life is a very convenient and environmentally friendly way of disposal, but some medical devices are also expected to degrade in biological environments, often in vivo. Since the main connecting linkage of PHB chains is the ester bond, one needs a catalyst initiating ester hydrolysis for efficient degradation. Hydrolytically this reaction is generally catalyzed in acidic or basic media by protons or hydroxide ions.

Because of its importance, quite a few studies have been published on the hdyrolitic degradation of PHB. De Roo [25] and Braunegg [26], for example, used acidic hydrolysis and methanolysis, respectively, for the degradation of PHB. Others, like Foster [27], Holland [28], and Saeki [29] degraded PHB by basic catalysts. The various methods yielded different products, both the monomer and longer degradation products were detected among the products, but racemization was also observed.

Enzymatic reactions are very specific and well defined thus the use of a PHB hydrolase enzyme natively synthetized by prokaryotes (bacteria) or eukaryotes (fungi) [30] would result in the more efficient degradation of the polymer. Several papers reported the production and characterization of extracellular PHB hydrolase enzymes produced by various bacterial strains, such as *Alcaligenes faecalis* [31, 32], *Comamonas testosteroni* [33], *Pseudomonas lemoignei* [34], *Pseudomonas pickettii* [35], while others used enzymes natively produced by fungi (*Fusarium solani* [36], *Paecilomyces lilacinus* [37], *Penicilium*

funiculosum [38]). As extracellular enzymes are secreted by the organism, cell disruption is not required as an intermediate step in protein production. Besides the simplicity of their synthesis, extracellular enzymes are usually not very sensitive to environmental factors like high temperature, acidic or basic pH, as well as the presence of oxidizing or reducing agents. Besides the monomer, these extracellular enzymes usually produce longer metabolites [39], while one of them natively produced by the strain *Paucimonas lemoignei* yielded predominantly oligomers [39]. On the other hand, as shown by Chen et al. [39], the novel intracellular enzyme natively produced by *Bacillus megaterium* yielded solely (R)-3-hydroxybutyric acid molecules in the controlled degradation of PHB.

The first step of every heterogeneous reaction catalyzed by an enzyme is its adsorption on the surface of the polymer, and adsorption usually determines the overall rate of the reaction. Accordingly, enzyme adsorption and the following reaction are widely studied and used in practice, like for the decomposition of hazardous or even toxic dyes [40-50], or for the complete removal of toxic dyes from wastewater [51-58]. Adsorption-based methods make possible the removal [59] and decomposition [60-67] of a large number of toxic organic compounds mainly produced by the textile industry [62-64]. Besides the removal of dyes and further hazardous organic byproducts, heterogeneously catalyzed enzyme reactions can be applied for the removal of non-organic contaminations, e.g. heavy metal ions from aqueous media [68-74], and the technique developed was used for the cleaning of wastewater [75-76]. As shown by Gupta, adsorption based methods can be utilized also for analytical purposes, for the determination of the concentration of metal ions [77] and organic compounds [78]. The number of methods applying heterogeneous catalytic reactions clearly indicate that these techniques are gaining more and more attention, but their practical implementation inevitably requires the knowledge of the kinetics of the related processes (adsorption, reaction), which is needed for the proper planning of a

reaction at industrial scale.

The same applies to the heterogeneous, enzyme-catalyzed degradation of PHB. The kinetics of degradation is at least as important for most practical purposes, as the outcome of the reaction itself. Composting technology has well-defined cycle times, while in vivo degradation of medical devices requires the exact knowledge of degradation rate. Although a number of papers have been published on enzyme-catalyzed heterogeneous reactions, which describe the rate of degradation as a function of enzyme concentration, like those of Mukai [79], Scandola [80], or Timmins [81], very little is known about the time dependence of heterogeneous enzyme reactions. The kinetics of enzyme-catalyzed hydrolysis is often described with the model proposed by Michaelis and Menten in 1913 [82]. However, the model was developed and is valid only for homogeneous reactions, but in practice, either in composting or in medical applications, degradation takes place in a heterogeneous medium thus the original model must be modified accordingly.

Besides the characterization and quantitative description of the degradation of PHB, the proper control of the reaction also offers the possibility of producing (R)-3-hydroxybutyric acid (3-HBA) in large purity. The production of 3-HBA is severely limited by the difficulties of chemical synthesis [83,84], in spite of the fact that the chiral compound is valuable biotechnologically and important in several application areas. It can be used for the synthesis of carbepenem antibiotics [85], as chiral building block for the production of macrolides (e.g. pyrenophorin, colletodiol and grahamimycin) [86,87] or for the synthesis of beta-lactones through 3-HBA dioxanone enolates [88]. 3-HBA is the normal component of blood and is one of the three ketones produced endogenously by ketogenesis [89]. 3-hydroxybutyric acid is a novel nutrition source due to its good penetration and rapid diffusion into peripheral tissues [90], can be used as a novel drug delivery system [91] or for the preparation of various modified 3-HBA based macromolecular chains [92].

It is clear that the biodegradation of PHB is very interesting and important for theory and practice alike. Accordingly, the goal of this work was the study of the kinetics of the enzyme-catalyzed degradation of the polymer, the analysis of the composition of the reaction product, and the quantitative description of degradation kinetics. A new model is proposed for kinetic analysis, which, unlike most available models, takes into account the heterogeneous nature of degradation. The possibility of using the reaction and its product in practice was also explored.

2. EXPERIMENTAL

2.1 Materials

Poly(3-hydroxybutyrate) granules were obtained from Metabolix Ltd. (Mirel M2100, ≥99,5% purity) with an approximate crystallinity of 60%. HIS-tagged poly(3-hydroxybutyrate) depolymerase enzyme molecules were produced by recombinant *Escherichia Coli* bacteria [strain: Origami DE3 (Novagen), plasmid: pGS1865 bearing the depolymerase gene of the bacteria *Bacillus Megaterium*] purified by affinity chromatography on a Ni-nitrilotriacedic acid (NTA) agarose column.

2.2 Sample preparation

Amorphous poly(3-hydroxybutyrate) films were prepared by compression molding and solvent casting, respectively. Films of 100 μm thickness were compression molded using a Fontijne SRA 100 machine at 120 kN, 3 min, 220 °C and at a cooling rate of about 30 °C/min. Films were cast onto a glass surface from a chloroform solution of 2 m/m% of the polymer and subsequently kept at constant temperature (25 °C) and relative humidity (50 %).

2.3 Methods

The enzymatic degradation of amorphous poly(3-hydroxybutyrate) films was carried out in Erlenmeyer flasks, at 37 °C with continuous stirring at 200 rpm. The aqueous media consisted of 100 mmol/dm^3 NaCl and 20 mmol/dm^3 Tris/HCl buffer [tris(hydroxymethyl)-aminomethane hydrochloric acid salt] adjusted to pH 8.0. The amorphous polymer films and the enzyme solution were simultaneously added to the Erlenmeyer flasks, the latter in a quantity to provide 7 μg/ml enzyme concentration. The experimental conditions used (37 °C temperature, pH 8.0 basicity, 7 μg/ml enzyme concentration) were selected from previously published papers reporting the dependence of maximum enzyme activity on these variables [79, 80,84].

Enzymatic degradation was followed with UV-VIS spectrophotometry (UNICAM UV-500, wavelength range: 200-300 nm) and HPLC chromatography (Merck-Hitachi LaChrom Elite, equipped with a DAD detector set for the same 200-300 nm wavelength range). The HPLC column (LiChroChart 250-4) contained LiChrospher 100 RP-18 type end-capped silica (5 μm average pore size), the eluent was a phosphate buffer of 10 mmol/dm^3 at pH 3.0. Both the UV-VIS spectra and the HPLC chromatograms were recorded with a time interval of 20 min over 3 hours. Besides repetitive sampling, UV-VIS measurements were carried out also with an online UV-VIS spectrophotometer (PharmaTest PTWS 600) in order to measure the time dependent spectrum of the degrading medium, which is not biased by intermittent sampling. The composition of the samples degraded for various times were analyzed also by mass spectrometry (TA Instruments SDT 2960 MS).

3. RESULTS AND DISCUSSION

The results of the study are reported in several sections; data obtained on the kinetics of enzymatic degradation are reported in the first. The kinetic model developed for the

quantitative analysis of degradation kinetics is presented in the next one followed by the estimation of parameters and the discussion of the results including their practical relevance.

3.1. Degradation kinetics

UV-VIS spectra recorded on the degradation media after various times are presented in **Fig. 1**. The absorption peak appearing at around 215 nm in the UV-Vis spectra was assigned by several researchers [79,80,84] to the specific absorption of the monomer, 3-hydroxybutiric acid. Peak maximum was found to shift slightly towards smaller wavelengths; maximum absorbance was always determined at the corresponding wavelength of the maximum and not at 215 nm as done by several groups [79,80,84]. The shift in the position of the absorbance peak might be caused by a number of effects; one of them is the formation of metabolites with a specific UV absorbance close to that of the monomer (~215 nm). The UV spectra of different metabolites, which might form during degradation, is expected to be very similar to that of the monomer that makes quantitative analysis quite difficult. Maximum absorbance measured on both compression molded and solvent cast films is plotted against the time of degradation in **Fig. 2**.

In order to separate the components of the solution obtained after various times of degradation, samples were injected onto an inverse phase liquid chromatograph. The chromatograms recorded on various samples are presented in **Fig. 3**. The first peak appearing at 100 s belongs to the Tris/HCl buffer cation; its concentration remained constant (10 mmol/dm^3) in the entire timespan of the degradation experiment. The height of the second peak, however, depends on time proving the formation of increasing amounts of a product, possibly the monomer. The lack of additional peaks indicates that longer metabolites do not form or the HPLC column cannot separate them from the monomer.

To exclude the latter possibility, MS spectra were recorded at the end of the

degradation period, after 3 hours (**Fig. 4**). The only peak of relevance appears at 103.15 g/mol. The molar mass of 3-hydroxybutyric acid is 104.11 g/mol, but at pH 8.0 practically all molecules of the weak acid with the pK_a value of 4.8 are present in ionized form. Deprotonation is expected to decrease the molar mass of the dimer [3-(3-hydroxybutanoyl) oxybutanoic acid] as well, and thus its presence should have appeared as a peak at 189.22 g/mol. Because of the lack of any peaks in this range or above, one may safely state that the only product of the enzymatic degradation is the monomer.

The results presented above clearly show the general tendency of the kinetics of enzymatic degradation, but the plotted values (**Figs. 2** and **4**) are inevitably biased by systematic and stochastic errors caused by the nature of repetitive intermittent sampling. In order to minimize these errors, additional samples were prepared and their degradation monitored with a fully automated on-line UV-VIS measurement system. The results of the measurements are plotted in **Fig. 5** and they confirm those obtained by recursive sampling. Compression molded films degrade faster than those prepared by solvent casting, and degradation proceeds through an initial accelerating stage to achieve constant rate later. Automated sampling carried out by a software-controlled peristaltic pump has the undeniable advantage of providing the quasi-continuous flow of the aqueous medium through the measurement cell, but certain issues may arise about the accuracy of UV detection also here. The online UV-VIS spectrophotometer requires an initially specified wavelength, at which absorbance is recorded continuously as a function of time during the measurement. This wavelength was set to 215 nm, resulting in a systematic error, since the position of maximum absorption shifts towards smaller wavelengths with increasing degradation time (see **Fig. 1**).

3.2 The model

Figs. 2 and **6** shows that the rate of degradation initially increases and eventually, after 40-60 min, it becomes constant. The linearity of the correlations reveals that degradation carried out both on compression molded and solvent cast films proceeds similarly with a constant rate. An appropriate kinetic model may help to explain the initial accelerating stage.

Among many others, Michaelis and Menten [82] proposed a model to describe the kinetics of enzymatic reactions. In their original publication, they presented a two-step model, which included the formation of an enzyme-substrate complex first, and then its subsequent decomposition. This latter step may yield an unmodified substrate or a product molecule. Neither of them modifies the enzyme, the protein remains intact in both cases. The model is usually expressed as

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \mathrm{ES} \xrightarrow{k_c} \mathrm{E} + \mathrm{P} \qquad (1)$$

where E is the enzyme, S the substrate, ES the activated complex, and P the product. Each of the reaction steps has its own rate constant, i.e. k_1, k_{-1} and k_c. The determination the rate constants requires the knowledge of the concentration of the components and the kinetical order or the reaction, which, according to the original article, corresponds to the number of reactants participating in the respective reaction step.

According to these assumptions, the concentration of the enzyme increases by the decomposition of the activated complex and decreases by the formation of the ES complex

$$\frac{\mathrm{d}[E](t)}{\mathrm{d}t} = -k_1[E](t)[S](t) + k_{-1}[ES](t) + k_c[ES](t) \qquad (2)$$

The reaction is calculated similarly for the ES complex; its concentration increases by its formation and decreases by its decomposition, i.e.

$$\frac{\mathrm{d}[ES](t)}{\mathrm{d}t} = k_1[E](t)[S](t) - k_{-1}[ES](t) - k_c[ES](t) \qquad (3)$$

The concentration of the substrate changes only in two reactions, in the formation of the ES complex and during its decomposition

$$\frac{\mathrm{d}[S](t)}{\mathrm{d}t} = -k_1[E](t)[S](t) + k_{-1}[ES](t) \qquad (4)$$

Finally, the rate of the formation of the product molecules is affected only by the concentration of the ES complex

$$\frac{\mathrm{d}[P](t)}{\mathrm{d}t} = k_c[ES](t) \qquad (5)$$

Although the Michaleis-Menten model described above is quite simple, preliminary calculations based on its differential equations (**Eqs. 2-5**) usually provide surprisingly exact results. In our case, however, the model has to be modified to take into account the heterogeneous character of enzyme catalyzed hydrolysis.

The first modification to be made is related to the formation of the enzyme-substrate complex. In the degradation of polyhydroxyalkanoates the substrates are ester groups and only those located at the surface of the polymer film are able to participate in the reaction and form an activated complex. The approximate diameter of a PHB depolymerase molecule is about 8 ± 3 nm [93], which makes its diffusion into the polymer

phase practically impossible. As the surface of the PHB film placed into the aqueous media remains constant throughout the 3 hours of the measurement, the number of ester groups located on the surface can be assumed constant as well.

The adsorption kinetics of the enzyme molecules onto the surface of the film must be also considered and accommodated into the model. Since the formation of an activated complex requires a free enzyme molecule and a free ester group on the surface, only a monomolecular layer of the enzyme can be active and catalyze the degradation reaction [93]. Accordingly, the total amount of active enzyme molecules adsorbed on the surface of the polymer is rather small and thus enzyme concentration ([*E*]) is regarded as constant in the model.

The application of the modifications described above leads to the following equations

$$\frac{\mathrm{d}[E](t)}{\mathrm{d}t} = 0 \tag{6}$$

$$\frac{\mathrm{d}[S](t)}{\mathrm{d}t} = 0 \tag{7}$$

$$\frac{\mathrm{d}[ES](t)}{\mathrm{d}t} = k_1\, E_0\, S_0 - k_{-1}[ES](t) - k_c[ES](t) \tag{8}$$

$$\frac{\mathrm{d}[P](t)}{\mathrm{d}t} = k_c[ES](t) \tag{9}$$

where E_0 and S_0 are the constant number of enzyme molecules and ester groups located on the surface of the polymer film, respectively.

3.3 Application of the model, parameters

The differential equation system presented above (**Eqs. 8** and **9**) must be solved in order to obtain the unknown [*ES*](*t*) and [*P*](*t*) functions. The analytical solution obtained takes the following form

$$[ES](t) = C\exp[(-k_{-1}+k_c)t] + k_1 E_0 S_0 \frac{1}{k_{-1}+k_c} \qquad (10)$$

$$[P](t) = C\frac{-k_c}{k_{-1}+k_c}\exp[-(k_{-1}+k_c)t] + k_c\frac{k_1 E_0 S_0}{k_{-1}+k_c}t + C' \qquad (11)$$

The graphical form of the functions expressed by **Eqs. 10** and **11** are plotted in **Fig. 6**, which shows the concentration of the activated complex ([*ES*]) and that the product ([*P*]) as a function of time as predicted by the new model. According to the model derived above, the total amount of enzyme molecules adsorbed on the polymer surface approaches a constant value. Doi and his colleagues [93] studied the adsorption kinetics of several PHB depolymerase enzymes, and although none of them was the strain Bacillus Megaterium, the obtained enzyme-substrate complex vs. time plots were quite similar to that predicted by our model (**Fig. 6**). Accordingly, product concentration goes through an initial accelerating phase with increasing adsorption of enzyme molecules, but as the total number of ES complexes reaches its maximum, the formation rate of product molecules also converges to a constant value, i.e. the product concentration vs. time function becomes linear.

While the concentration of the ES complex was not measured in our recent study, product formation was monitored with several independent methods. In order to be able to compare measured data with the prediction of the model, absorbance values must be converted to concentrations, and the model equation (**Eq. 11**) fitted to the measured data. The conversion was done by calibration using commercial 3-hydroxybutiric acid, while the

fitting was carried out with a nonlinear iterative method using the Levenberg-Marquardt algorithm.

In order to facilitate the fitting procedure and the determination of the constants of the model, **Eqs. 10** and **11** are further simplified. The merging of the rate constants k and the integration constant C, as well as introducing parameter λ in the form $\lambda = k_{-1} + k_c$, simplifies the exponential part of **Eq. 11** and the linear term can be modified in a similar way as well. Taking into consideration the initial condition of the model, i.e. $[P](0) = 0$, shows that the C' constant equals the preexponent (A) of the simplified equation. All these simplifications result in the final form of the equation, which can be used for fitting, and the estimation of the parameters, i.e.

$$[P](t) = A\exp(\lambda t) + p\,t - A \qquad (12)$$

where λ indicates the time necessary to reach the stationary state of the degradation reaction, p is the formation rate of the product molecules (monomer) and thus the rate of degradation in the stationary stage, while A is the nominal amount of monomer formed in the first, nonlinear stage.

The simplified formula was fitted to the experimental results obtained both by intermittent sampling and on/line UV-VIS detection. The results of the fitting procedure are shown in **Figs. 7** and **8**, respectively. The fit is excellent in each case proving that the model is adequate for the description of the kinetics of the enzymatic degradation of PHB, but most probably also for that of other aliphatic polyesters. The comparison of the measured and predicted data also shows, especially for films prepared by compression molding that the rate of degradation decreases at longer times, deviates from the predicted line. A probable reason of the deviation is the denaturation of the enzyme, but this tentative explanation

needs further study and proof. Another interesting phenomenon is the slower degradation of solvent cast films. Only tentative explanations can be given here too. Although both were amorphous, the morphology of the two films might be different, compression molding at high temperature might result in some degradation, and finally the solvent used for casting might not have been removed completely and could have led to the denaturation of the enzyme. Although a final and unambiguous explanation cannot be given for these phenomena, the new model may help considerably the identification and the quantitative determination of the effect of factors influencing the enzymatic degradation of aliphatic polyesters.

The fitting of the model to the experimental data yielded also numerical values for the parameters of **Eq. 12**, which are listed in **Table 1**. The parameters clearly show, as mentioned above, that the degradation of solvent cast films is generally slower than that of the compression molded ones; the rate of the second, stationary stage (p) is significantly slower for solvent cast than for compression films and 3-hydroxybutyric acid concentration in the first, nonlinear phase (A) is also smaller. The attention must be called here to the fact that parameters determined by different detection methods, i.e. intermittent and online, show excellent agreement in spite of the inherent deficiencies of both techniques.

Besides reaction rates, parameter fitting provided valuable information about the adsorption of enzyme molecules as well. The larger absolute values of the time constant (λ) indicates that a shorter time is required to reach the equilibrium in adsorption in the case of compression molded than for solvent cast films. In the latter case, the nonlinear phase seems to be longer implying that the adsorption kinetics of enzyme molecules dependents on the method used to prepare the PHB films, probably on their morphology or surface quality.

4. CONCLUSIONS

Amorphous poly(3-hydroxybutyrate) films prepared by compression molding and solvent casting were degraded with the hydrolase enzyme natively synthetized by the strain *Bacillus megaterium*. The results showed that the enzyme catalyzes the degradation of PHB indeed. Degradation proceeds in two stages, an accelerating stage during which the enzyme adsorbs on the surface of the film, and a steady state with constant rate. Biodegradation produces the (R)-3-hydroxybutiric acid monomer in high purity, no metabolites or side products were detected in the degradation product. The kinetics of degradation was described quantitatively by a modified form of Michaelis-Menten model. Modifications had to take into account the heterogeneous nature of the degradation reaction. The new model assumes constant substrate and enzyme concentration, which simplifies treatment considerably. The parameters determined by the fitting of the model to the experimental values were consistent and did not depend on the method of detection (recursive or on-line). On the other hand the rate of degradation depended significantly on the technique used for the preparation of the films indicating parameters not accounted for during the study. The degradation of PHB by the strain used offers a simple way for the economic production of 3-hydroxybutyric acid, a compound used in chemical synthesis or as a component of biomedical systems.

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REFERENCES

1. Gandini, A., Lacerda, T. M., *Progress in Polymer Science* **48**, 1–39, (2015)
2. Noreen, A., Zia, K. M., Zuber, M., Ali, M., Mujahid, M., *International Journal of Biological Macromolecules* **86**, 937–949, (2016)
3. Long Yu, Katherine Dean, Lin Li, *Progress in Polymer Science* **31**, 576–602, (2006)
4. Fertier, L., Koleilat, H., Stemmelen, M., Giani, O., Joly-Duhamel, C., Lapinte, V., Robin, J. J., *Progress in Polymer Science* **38**, 932–962, (2013)
5. Fernández, K., Aburto, J., Plessing, C., Rockel, M., Aspé, E., *Food Chemistry* **207**, 75–85, (2016)
6. Djidi, D., Mignard, N., Taha, M., *Industrial Crops and Products* **72**, 220–230, (2015)
7. Han, X., Wang, D., Chen, X., Lin, H., Qu, F., *Materials Science and Engineering C* **43**, 367–374, (2014)
8. Belkhir, K., Shen, H., Chen, J., Jegat, C., Taha, M., *European Polymer Journal* **66**, 290–300, (2015)
9. Bishai, M., De, S., Adhikari, B., Banerjee, R., *European Polymer Journal* **54**, 52–61, (2014)
10. Zuo, Y., Gu, J., Yang, L., Qiao, Z., Tan, H., Zhang, Y., *International Journal of Biological Macromolecules* **64**, 174– 180, (2014)
11. Leong, Y. K., Show, P. L., Ooi C. W., Ling, T. C., Lan, J. C., *Journal of Biotechnology* **180**, 52–65, (2014)
12. Mengmeng, C., Hong, C., Qingliang, Z., Shirley, S. N., Jie, R., *Bioresource Technology* **100**, 1399–1405, (2009)
13. Ji, Q., Wang, H., Wang, X., *Bioresource Technology* **140**, 328–336, (2013)
14. Huschner, F., Grousseaua, E., Brigham, C. J., Plassmeier, J., Popovic, M., Rha, C.,

Sinskey, A. J., *Process Biochemistry* **50**, 165–172, (2015)

15. Ocampo-López, C., Colorado-Arias, S., Ramírez, M., *Journal of Applied Research and Technology* **13**, 498–503, (2015)
16. Castilho, L. R., Mitchell, D. A., Freire, D. M. G., *Bioresource Technology* **100**, 5996–6009, (2009)
17. Sun, Z., Ramsay, J. A., Guay, M., Ramsay, B., *Journal of Biotechnology* **132**, 280–282, (2007)
18. Prabisha, T. P., Sindhu, R., Binod, P., Sankar, V., Raghu, K. G., Pandey, A., *Biochemical Engineering Journal* **101**, 150–159, (2015)
19. Moreno, P., Yanez, C., Cardozo, N. S. M., Escalante, H., Combariza, M. Y., Guzman, C., *New Biotechnology* **32**, 682-689, (2015)
20. Rahnama, F., Vasheghani-Farahani, E., Yazdian, F., Shojaosadati, S. A., *Biochemical Engineering Journal* **65**, 51–56, (2012)
21. Naranjo, J. M., Posada, J. A., Higuita, J. C., Cardona, C. A., *Bioresource Technology* **133**, 38–44, (2013)
22. Reddy, M. V., Mawatari, Y., Yajima, Y., Seki, C., Hoshino, T., Chang, Y., *Bioresource Technology* **192**, 711–717, (2015)
23. Arifin, Y., Sabri, S., Sugiarto, H., Krömer, J. O., Vickers, C. E., Nielsen, L. K., *Journal of Biotechnology* **156**, 275–278, (2011)
24. Chatzidoukas, C., Penloglou, G., Kiparissides, C., *Biochemical Engineering Journal* **71**, 72–80, (2013)
25. De Roo, G., Kellerhals, M. B., Ren, Q., Witholt, B., Kessler, B., *Biotechnology and Bioengineering* **77**, 717–722, (2002)
26. Braunegg, G., Sonnleitner, B., Lafferty, R. M., *European journal of applied microbiology and biotechnology*, **6** 29–37, (1978)

27. Foster, L. J. R., Tighe, B. J., *Polymer Degradation and Stability* **87**, 1-10, (2005)

28. Holland, S. J., Jolly, A. M., Yasin, M., Tighe, B. J., *Biomaterials* **8**, 289-295, (1987)

29. Saeki, T., Tsukegi, T., Tsuji, H., Daimon, H., Fujie, K., *Polymer* **46**, 2157–2162, (2005)

30. Jendrossek, D., *Polymer Degradation and Stability* **59**, 317-325, (1998)

31. Tanio, T., Fukui, T., Shirakura, Y., Saito, T., Tomita, K., Kaiho, T., *European Journal of Biochemistry* **124**, 71-77, (1982)

32. Shirakura, Y., Fukui, T., Saito, T., Okamoto, Y., Narikawa, T., Koide, K., Tomita, K., Takemasa, T., Masamune, S., *Biochimica et Biophysica Acta* **880**, 46-53, (1986)

33. Kasuya, K., Doi, Y., Yao, T., *Polymer Degradation and Stability* **45**, 379-386, (1994)

34. Briese, B. H., Schmidt, B., Jendrossek, D., *Journal of Environmental Polymer Degradation* **2**, 75-87, (1994)

35. Shiraki, M., Shimada, T., Tatsumichi, M., Saito, T., *Journal of Environmental Polymer Degradation* **3**, 13-21 (1995)

36. Nakayama, K., Saito, T., Fukui, T., Shirakura, Y., Tomita, K., *Biochimica et Biophysica Acta* **827**, 63-72, (1985)

37. Oda, Y., Osaka, H., Urakami, T., Tonomura, K., *Current Microbiology* **34**, 230-232, (1997)

38. Brucato, C. L., Wong, S. S., *Archives of Biochemistry and Biophysics* **290**, 497-502, (1991)

39. Chen, H., Pan S., Shaw, G., *Applied and Environmental Microbiology* **75**, 5290–5299, (2009)

40. Gupta, V. K., Jain, R., Mittal, A., Saleh, T. A., Nayak, A., Agarwal, S., Sikarwar, S., *Materials Science and Engineering: C* **32**, 12-17, (2012)

41. Saleh, T. A., Gupta, V. K., *Journal of Colloid and Interface Science* **371**, 101-106, (2012)

42. Rajendran, S., Khan, M. M., Gracia, F., Qin, J. Q., Gupta, V. K., Arumainathan, S., *Scientific Reports* **6**, 1-11, (2016)

43. Saravanan, R., Sacari, E., Gracia, F., Khan, M. M., Mosquera, E., Gupta, V. K., *Journal of Molecular Liquids* **221**, 1029-1033, (2016)

44. Saravanan, R., Gupta, V. K., Mosquera, E., Gracia, F., Narayanan, V., Stephen, A., *Journal of Saudi Chemical Society* **19**, 521-527, (2015)

45. Saravanan, R., Gracia, F., Khan, M. M., Poornima, V., Gupta, V. K., Narayanan, V., Stephen, A., *Journal of Molecular Liquids* **209**, 374-380, (2015)

46. Saravanan, R., Gupta, V. K., Mosquera, E., Gracia, F., *Journal of Molecular Liquids* **198**, 409-412, (2014)

47. Saravanan, R., Gupta, V. K., Narayanan, V., Stephen, A., *Journal of Molecular Liquids* **181**, 133-141, (2013)

48. Saravanan, R., Joicy, S., Gupta, V. K., Narayanan, V., Stephen, A., *Materials Science and Engineering: C* **33**, 4725-4731, (2013)

49. Saravanan, R., Karthikeyan, S., Gupta, V. K., Sekaran, G., Narayanan, V., Stephen, A., *Materials Science and Engineering: C* **33**, 91-98, (2013)

50. Saleh, T. A., Gupta, V. K., *Journal of Colloid and Interface Science* **362**, 337-344, (2011)

51. Mittal, A., Mittal, J., Malviya, A., Kaur, D., Gupta, V. K., *Journal of Colloid and Interface Science* **342**, 518-527, (2010)

52. Mittal, A., Kaur, D., Malviya, A., Mittal, J., Gupta, V. K., *Journal of Colloid and Interface Science* **337**, 345-354, (2009)

53. Mittal, A., Mittal, J., Malviya, A., Gupta, V. K., *Journal of Colloid and Interface Science* **340**, 16-26, (2009)

54. Mittal, A., Mittal, J., Malviya, A., Gupta, V. K., *Journal of Colloid and Interface Science* **344**, 497-507, (2010)

55. Gupta, V. K., Jain, R., Nayak, A., Agarwal, S., Shrivastava, M., *Materials Science and Engineering: C* **31**, 1062-1067, (2011)

56. Jain, A. K., Gupta, V. K., Bhatnagar, A., Suhas, *Separation Science and Technology* **38**, 463-481, (2003)

57. Gupta, V. K., Kumar, R., Nayak, A., Saleh, T. A., Barakat, M. A., *Advances in Colloid and Interface Science* **193-194**, 24-34, (2013)

58. Gupta, V. K., Mittal, A., Jhare, D., Mittal, J., *RSC Advances* **2**, 8381-8389, (2012)

59. Gupta, V. K., Saleh, T. A., *Environmental Science and Pollution Research* **20**, 2828-2843, (2013)

60. Karthikeyan, S., Gupta, V. K., Boopathy, R., Titus, A., Sekaran, G., *Journal of Molecular Liquids* **173**, 153-163, (2012)

61. Saravanan, R., Khan, M. M., Gupta, V. K., Mosquera, E., Gracia, F., Narayanan, V., Stephen, A., *RSC Advances* **5**, 34645-34651, (2015)

62. Saravanan, R., Mansoob Khan, M., Gupta, V. K., Mosquera, E., Gracia, F., Narayanan, V., Stephen, A., *Journal of Colloid and Interface Science* **452**, 126-133, (2015)

63. Saravanan, R., Gupta, V. K., Narayanan, V., Stephen, A., *Journal of the Taiwan Institute of Chemical Engineers* **45**, 1910-1917, (2014)

64. Saravanan, R., Karthikeyan, N., Gupta, V. K., Thirumal, E., Thangadurai, P., Narayanan, V., Stephen, A., *Materials Science and Engineering: C* **33**, 2235-2244, (2013)

65. Saravanan, R., Thirumal, E., Gupta, V. K., Narayanan, V., Stephen, A., *Journal of Molecular Liquids* **177**, 394-401, (2013)

66. Saravanan, R., Gupta, V. K., Prakash, T., Narayanan, V., Stephen, A., *Journal of Molecular Liquids* **178**, 88-93, (2013)

67. Mohammadi, N., Khani, H., Gupta, V. K., Amereh, E., Agarwal, S., *Journal of Colloid and Interface Science* **362**, 457-462, (2011)

68. Saleh, T. A., Gupta, V. K., *Environmental Science and Pollution Research* **19**, 1224-1228, (2012)

69. Gupta, V. K., Srivastava, S. K., Mohan, D., Sharma, S., *Waste Management* **17**, 517-522, (1998)

70. Gupta, V. K., Agarwal, S., Saleh, T. A., *Journal of Hazardous Materials* **185**, 17-23, (2011)

71. Gupta, V. K., Nayak, A., *Chemical Engineering Journal* **180**, 81-90, (2012)

72. Gupta, V. K., Nayak, A., Agarwal, S., *Environmental Engineering Research* **20**, 1-18, (2015)

73. Saravanan, R., Prakash, T., Gupta, V. K., Stephen, A., *Journal of Molecular Liquids* **193**, 160-165, (2014)

74. Saleh, T. A., Gupta, V. K., *Separation and Purification Technology* **89**, 245-251, (2012)

75. Gupta, V. K., Ali, I., Saleh, T. A., Nayak, A., Agarwal, S., *RSC Advances* **2**, 6380-6388, (2012)

76. Ahmaruzzaman, M., Gupta, V. K., *Industrial & Engineering Chemistry Research* **50**, 13589-13613, (2011)

77. Khani, H., Rofouei, M. K., Arab, P., Gupta, V. K., Vafaei, Z., *Journal of Hazardous Materials* **183**, 402-409, (2010)

78. Devaraj, M., Saravanan, R., Deivasigamani, R., Gupta, V. K., Gracia, F., Jayadevan, S., *Journal of Molecular Liquids* **221**, 930-941, (2016)

79. Mukai, K., Yamada, K., Doi, Y., *International Journal of Biological Macromolecules* **15**, 361-366, (1993)

80. Scandola, M., Focarete, M. L., Frisoni, G., *Macromolecules* **31**, 3846-3851, (1998)

81. Timmins, M. R., Lenz, R. W., *Polymer* **38**, 551-562, (1997)

82. Michaelis, L., Menten, M. L., *Biochemische Zeitschrift* **49**, 333-369, (1913)

83. Jaipuri, F. A., Jofre, M. F., Schwarz, K. A., Pohl, N. L., *Tetrahedron Letters* **45**, 4149–4152, (2004)

84. Beilstein, F.: Handbuch der organischen Chemie, 3. Auflage, 1. Band, Verlag Leopold Voss, 561-562, (1893)

85. Chiba, T., Nakai, T., *Chemistry Letters* **14,** 651–654, (1985)

86. Seebach, D., Chow, H. F., Jackson, R. F. W., Sutter, M. A., Thaisrivongs, S., Zimmermann, J., *Liebigs Annalen der Chemie* **7**, 1281–1308, (1986)

87. Seebach, D., Roggo, S., Zimmermann, J., In: Bartmann, W., Sharpless, K. B., (editors): Stereochemistry of organic and bioorganic transformation, Wiley–VCH, Weinheim, 85–126, (1987)

88. Lee, S. Y., Park, S. H., Lee, Y., Lee, S. H., In: Doi, Y, Steinbüchel, A., (editors) Biopolymers, polyesters III. Wiley–VCH, Weinheim, 375–387, (2002)

89. Pouton, C. W., Akhtar, S., *Advanced Drug Delivery Reviews* **18**, 133-162, (1996)

90. Tasaki, O., Hiraide, A., Shiozaki, T., Yamamura, H., Ninomiya, N., Sugimoto, H., *Journal of Parenteral and Enteral Nutrition* **23**, 321–325, (1999)

91. Kamachi, M., Zhang, S. M., Goodwin, S., Lenz, R. W., *Macromolecules* **34**, 6889–6894, (2001)

92. Song, J. J., Zhang, S. M., Lenz, R. W., Goodwin, S., *Biomacromolecules* **1**, 433–439,

(2000)

93. Kasuya, K., Inoue, Y., Doi, Y., *International Journal of Biological Macromolecules* **19**, 35-40, (1996)

Table 1 Parameters characterizing the enzymatic degradation of PHB obtained by the fitting of the new kinetic model to experimental data

Preparation method	Parameter	Detection method	
		Recursive sampling	Online measurement
Compression molding	p (mmol/dm^3/min)	0.0241	0.0244
	λ (1/min)	-0.0302	-0.0237
	A (mmol/dm^3)	0.8311	0.9890
Solvent casting	p (mmol/dm^3/min)	0.0133	0.0168
	λ (1/min)	-0.0249	-0.0223
	A (mmol/dm^3)	0.6648	0.8043

CAPTIONS

Fig. 1 UV-VIS spectra recorded on aqueous media containing compression molded films degrading for various length of times.

Fig. 2 Degradation kinetics of poly(3-hydroxybutyrate) films determined after intermittent sampling by UV-VIS spectroscopy. Symbols: (○) compression molding, (□) solvent casting.

Fig. 3 Chromatograms recorded on aqueous media after various times of degradation on compression molded PHB film.

Fig. 4 Mass spectrum of a reference sample containing 100 mmol/dm^3 3-hydroxybutyric acid and that recorded after 3 hour degradation on an aqueous solution containing a compression molded film.

Fig. 5 Online UV-VIS monitoring of the enzymatic degradation of PHB at 215 nm wavelength.

Fig. 6 Changing concentration of the intermediate complex ([*ES*]) and that of the product ([*P*]) as a function of time as predicted by the model modified for heterogeneous reaction (**Eqs. 10** and **11**).

Fig. 7 Fitting of the model to the kinetics of enzymatic degradation of PHB films. Detection was recursive UV-VIS spectroscopy.

Fig. 8 Fitting of the kinetic model of enzymatic degradation onto the data obtained by online UV-VIS measurements.