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Calculation of Carbon Balances for Evaluation of the Biodegradability of Polymers*

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Establishing carbon balances has been proven to be an applicable and powerful tool in testing biodegradability of polymers. In controlled degradation tests at a 4-L scale with the model polymer poly(β-hydroxybutyrate) (PHB), it was shown that the degree of degradation could not be determined with satisfactory accuracy from CO_2 release alone. Instead, the course of degradation was characterized by means of establishing carbon balances for the degradation of PHB with *Acidovorax facilis* and a mixed culture derived from compost. Different analytical methods for determining the different carbon fractions were adapted to the particular test conditions and compared. Quantitative determination of biomass and residual polymer were the main problems in establishing carbon balances. Amounts of biomass derived from protein measurements depend strongly on assumptions of the protein content of the biomass. Selective oxidation of biomass with hypochlorite was used as alternative, but here problems arose from insoluble metabolic products. Determination of soluble components with the method of chemical oxygen demand (COD) also includes empirical assumptions but seems acceptable if the dissolved carbon fraction is in the range of some 10% total carbon. Results confirm both analytical assays and theoretical approaches, in ending up at values very close to 100%, within an acceptable standard deviation range under test conditions comparable to standard test practice.

KEY WORDS: Biodegradation; poly(β-hydroxybutyrate); testing; carbon balance; carbon dioxide evolution.

INTRODUCTION

Scientific interest in biodegradable polymers has grown dramatically during the last few years, mainly for ecological reasons, waste deposit limitation being the most important [1, 2]. Research covers a wide range of specifications, e.g., the development of new materials synthesized by means of biotechnology [3–5] and classic organic chemistry [6] and improvement of material properties, including degradability pathways and patterns [7–9] as well as mechanical and physical properties [10–12], with much interest in the investigation of biodegradability itself, searching for measuring techniques and assays [13].

Dissimilation of an organic substrate to carbon dioxide is the main process of a biological degradation, at least under aerobic conditions. Thus, it presents itself as an ideal parameter for monitoring a biodegradation process and is used widely in research and standard testing [14]. A standardized test like the Sturm test [15] appoints a limit of 60% of theoretical evolution of carbon dioxide for proving biodegradation. However, carbon dioxide output from different substrates shows wide deviations related to specific formation of biomass and metabolic side products. From that, it was concluded that CO_2 evolution alone is not a satisfactory parameter to determine the degree of biodegradation of a polymer. Consequently a complete balance, in particular of carbon, including the detection of gaseous, soluble, and solid products must be established to demonstrate biodegradability. Tests, such as those described earlier, do

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not include such a total quantitative balance of utilization of polymer substrates [16].

Therefore, the aim of the present work was to calculate carbon balances for a polymer degradation process, with regard to tests procedures that are used in standard tests. In accordance with the well-known "Sturm test," a degradation process under controlled conditions was performed with the model polymer poly(β-hydroxybutyrate) (PHB); the course of degradation was followed by measuring carbon balances. Carbon balances were established using different analytical procedures and presumptions to minimize standard deviations.

Detection of gaseous components requires only the measurement of carbon dioxide, in principle, if a controlled oxygen supply guarantees aerobic conditions. Soluble products, oligomers of different molecular size, intermediates, and proteins secreted from the microbial cells were measured as chemical oxygen demand and by a protein assay. Solid products and polymer remnants required a combination of procedures to separate and detect different fractions.

PHB was used as a model substrate because of its ideal properties. It is known to be completely biodegradable and degradation pathways are well established [3]. PHB can be obtained as a homogeneous, thermoplastic material without any additives and the time frame of degradation allows analytical methods to be developed within a reasonable time scale.

EXPERIMENTAL

Polymer Substrate

A homopolymer of poly(3-hydroxybutyrate) was used as a model polymer. The polymer (PHB BX G08) was obtained as powder from ZENECA (Billingham, UK) and was characterized as follows: molar mass, 5.4 $\times$ 10^5 g/mol (according to the producer); M_η, 3.8 $\times$ 10^5 g/mol ($[\eta]_{\text{chloroform},25°C}$: 263 ml/g); stoichiometric carbon content, 55.8%; and surface area of particles from nitrogen adsorption measurements, 4.4 m^2/g.

Test Medium

Synthetic mineral salt medium for testing PHB biodegradation was prepared according to DIN 53739 as described earlier [17]; the pH was adjusted to 7.0 at 25°C. No additional carbon source (besides PHB) was contained in this medium.

Bacterial Strains and Inoculation

A PHB-decomposing bacterial isolate, screened in our laboratory, was used for the degradation experiments and was compared with a mixed culture derived from compost (compost plant Watenbüttel, Braunschweig, Germany).

The pure culture was obtained by isolation of a bacterial strain from sewage sludge as described earlier [18] and determined as *Acidovorax facilis* by the German Collection of Microorganisms and Cell Cultures (DSM, Braunschweig, Germany). *Acidovorax facilis* was precultured in nutrient-broth medium at 30°C and 150 rpm on a shaker for 24 h. Cells were separated by centrifugation, washed with sterile phosphate buffer and resuspended in 50 ml mineral salt medium, described above. The total cell number of the inoculum was determined in a Thoma counting chamber.

The compost-derived inoculum was prepared by suspending 50 g of postactive compost in 500 ml of a diluted Ringer solution (Merck 15525; 1 tablet in 500 ml of deionized water). After stirring the mixture for 60 min at room temperature, the suspension was filtered through a coarse-porous paper filter. The eluate was aerated immediately until use.

Analytical Means and Assays

Gravimetry

Dry weights were determined after centrifugation (6°C, 10,000*g*, 20 min) and lyophilization or vacuum drying at 60°C for 24 h, respectively.

NaOCl Treatment

For the selective determination of biomass and residual polymer, respectively, the dry mass after degradation was treated with sodium hypochlorite solution (NaOCl) using the method of Williamson and Wilkinson [19] with some modifications. With this method biomass can be oxidized selectively. Dried pellets from centrifuged samples (50 ml) were mixed with 13.5 ml of water and 1.5 ml of concentrated hypochlorite solution [approximately containing 12% (w/w) of active chlorine]. After incubation for 30 min at room temperature, solid components were separated by centrifugation, washed six times with water, and dried under vacuum.

Optimal hypochlorite concentration and relation of hypochlorite to biomass and incubation time were determined in preliminary tests. It was ensured that this

procedure did not alter the mass of PHB significantly (maximum weight reduction was approximately 3%), although it is known that hypochlorite can reduce the molar mass of PHB [3].

Alternative tests using lysozyme and 0.5 *M* NaOH gave unsatisfactory separation of biomass and polymer.

Chemical Oxygen Demand (COD)

Supernatants of centrifuged samples were analyzed for COD, using a commercial test kit (Dr. Lang LCL 314, Düsseldorf, Germany). The measuring range was 15 to 150 mg O_2/L.

Protein Management

Proteins were determined according to the method of Lowry [20]. Calibration was performed with BSA in the range of 10–100 μg/ml. For the determination of intracellular proteins, the pellet obtained from centrifugation of the 10-ml sample was treated with 5 ml of 0.5 *M* NaOH at 60°C for 2 h. In this case, 0.5 *M* NaOH was used instead of water for dilution and calibration with bovine serum albumin (BSA) applying the Lowry method.

Recently, a modified method using a surfactant was published by Sperandio *et al.* [21]. This modified procedure was not implemented for this investigation, to keep a homogeneous treatment for all samples.

Gas Chromatography

In some cases during the degradation experiment, the concentration of residual PHB was determined using gas chromatography. A 10-ml sample was centrifuged, the pellet was washed with water and dried in vacuum, 2 ml of chloroform and 2 ml of a mixture of 50 mg sodium benzoate, 3 ml of concentrated H_2SO_4 and 97 ml of methanol were added, and the suspension was incubated for 2 h at 100°C. Then 1 ml water was added, the mixture was shaken, and the organic phase was used for gas chromatographic analysis (Chromatograph HP 5890 A; column HP 20 Carbowax 20M) [22, 23].

Carbon Dioxide

CO_2 determination by means of $BaCO_3$ precipitation was performed as described earlier [17]. Determination by infrared detection was carried out with an automatized analyzer (Servomex, Crowborough, UK). For calibration, nitrogen (0% CO_2) and a calibration gas (0.04% CO_2; Linde KG, München, Germany) were used. Carbon dioxide concentration (%) was reported as the average value over 2 h. For calculating the amount of carbon dioxide released, the exact flow rate was determined by means of a simple water replacement technique.

Performance of Degradation Experiments

The dimensions of the test equipment and the performance of the experiment were based on criteria and experiences from earlier investigations with a smaller "Sturm test" apparatus [17]. For investigations concerning carbon balances, the test system should be controllable and large enough to handle a number of samples with appropriate volumes for the different analytical procedures to be compared. Taking into account these requests, a 4-L lab-fermenter system was chosen for the degradation tests. Temperature, pH, agitation rate, and dissolved oxygen could be measured and controlled with this equipment.

In total, six degradation experiments (V1–V6) were performed. During the first two tests (V1 and V2), CO_2 was detected using $Ba(OH)_2$ solutions, as described in the OECD guidelines. Because the larger scale of the degradation system resulted in high gas flow rates, this system turned out to be inadequate, i.e., limited gas adsorption and leaks in the gas washing bottle due to the high pressure, resulted in insufficient accuracy in detecting CO_2. For this reason, an IR-carbon dioxide analyzer was used for the last four experiments (V3 to V6).

Along with modifications to the CO_2 detection system, the analytical procedures for determining biomass and residual polymer were adapted and improved during the first experiments. Thus, results from these tests were used only to improve the experimental and analytical procedures and, hence, are not presented.

Apparatus

Degradation experiments were performed in a 4000-ml fermenter (Setric Genie Industrial, France). The medium was thermostated to 25°C and the pH was kept at 7.0 by automatic addition of NaOH. Agitation by a mechanical inclined-blade stirrer initially was kept at 150 rpm but was regulated up to 470 rpm when the partial pressure of oxygen (pO_2) fell short of 25%. Pressurized air was used for aeration at a flow rate of 160–175 L/h (corresponding to 0.72–0.75 v/vm). Three gas washing bottles (1000 ml) filled with 800 ml of 5 *M* KOH, respectively, and one 1000-ml bottle filled with 800 ml of 0.0125 *M* $Ba(OH)_2$ as an indicator were used to remove CO_2 from the air.

The fermenter was filled with 3700 ml of medium and 1 ml of a silicon antifoaming agent and was subsequently autoclaved. After connecting the degradation vessel to the air supply and CO_2-detection module, PHB powder was added at a concentration of 1 g/L as substrate. The polymer had not been sterilized because thermal treatment would alter its properties and washing with alcohol led to an aggregation of the powder particles. Before inoculating the fermenter, the apparatus was purged with carbon dioxide-free air until no CO_2 could be detected with the infrared analyzer.

Microorganisms and Inoculation

Acidovorax facilis was chosen from a number of strains isolated from sewage sludge, soil, or compost eluates as described earlier [18]. It shows good growth, on both polymer as single carbon source and the usual nutrients as well, and was stable in biological (and degradative) properties for a series of experiments. An amount of cell suspension containing 1.9×10^{11} (corresponding to a cell number of 0.5×10^7 cells/ml in the degradation broth) was suspended in 140 ml mineral medium and immediately used for inoculation.

In addition, for one of the degradation experiments (V6), a mixed culture derived from a compost eluate was applied. It is important for practical testing of degradability to use a microbiological population as broad as possible. Only with mixed cultures can a satisfactory comparison of the test for different (polymer) materials be achieved.

The compost had already passed through the active phase with temperatures up to 60–70°C. To avoid a long lag phase of the test, which is run at 25°C, it is important to use no material of the active phase, where thermophilic microorganisms are predominant. A 1 vol% of eluate was used for inoculation, thus organic carbon material eluted from compost could be neglected in the test system used.

Processing/Downstream Processing

After inoculation, the carbon dioxide content of the air was monitored and averaged for 2-h intervals. At different times, depending on the course of degradation in terms of CO_2 evolution, samples of the fermentation broth were taken. Figure 1 shows the scheme of downstream processing and analysis performed with these samples. Problems generally can arise from sampling in heterogeneous systems. Nevertheless, the volumes used in these experiments were big enough to exclude significant errors due to sampling. In practice, carbon balances would be determined only once, at the end of the test, in which case problems such as these would not occur.

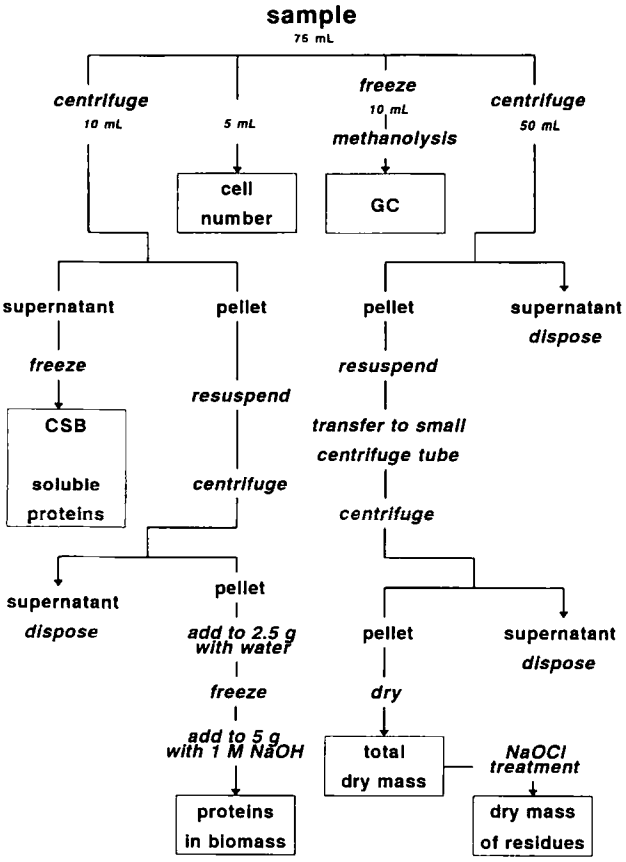

Fig. 1. Scheme of sample handling and analytical procedures.

To enhance the accuracy of the analytical results, especially of gravimetric measurements, it was important to minimize the number of sample transfers from one container to another.

RESULTS

The course of PHB degradation was monitored using different characteristic parameters. Analytical results of degradation tests V3–V6 are listed in Table I. Carbon dioxide evolution directly characterizes the degradative activity of the microorganisms. The volume of the samples and the exact remaining volume of the medium are important for calculating the carbon balances. The chemical oxygen demand represents the soluble

Table I. Experimental Data of Degradation Tests

	Time (h)							
V3: carbon introduced with PHB, 9702 mg[a]	5	19	26	43	69	91	116	
Carbon dioxide (Σ mg)	6.6	918	1898	3661	4328	4536	4761	
Volume before sampling (L)	3.39	0.06	3.26	3.20	3.13	3.06	2.99	
Sample volume (L)	0.07	134	0.065	0.065	0.07	0.07	0.07	
COD (mg O_2/L)	56	1075	144	n.d.	160[b]	155	80	
Total dry weight (mg/L)	1416	854	848	5.24	166	396	924	
Dry weight after NaOCl (mg/L)	1330	67	574	1.31	12	69	193[c]	
Proteins in solids (mg/L)	9	8	88	1.51	128	86	132	
Soluble proteins (mg/L)	2	n.d.	13	27	63[b]	55	43	
	Time (h)							
V4: carbon introduced with PHB, 7638 mg[a]	4	16	24	40	64	91		
Carbon dioxide (Σ mg)	6.2	572	1526	3577	4844	5144		
Volume before sampling (L)	3.61	3.53	3.38	3.23	3.08	2.93		
Sample volume (L)	0.08	0.15	0.15	0.15	0.15	0.15		
COD (mg O_2/L)	65[d]	70	70	116	139[b]	139		
Total dry weight (mg/L)	973	940	802	456	278	241		
Dry weight after NaOCl (mg/L)	842	620	160	30	n.d.	11		
Proteins in solids (mg/L)	7	47	112	265	234[e]	191[e]		
Soluble proteins (mg/L)	0	5	9	21	46[b]	55		
	Time (h)							
V5: carbon introduced with PHB, 6164 mg[a]	5	16	24	41	65	91	115	120
Carbon dioxide (Σ mg)	0	0	364	979	2906	3536	3755	3802
Volume before sample (L)	3.55	3.4	3.32	3.17	3.02	2.87	2.72	2.15
Sample volume (L)	0.15	0.08	0.15	0.15	0.15	0.15	0.15	0.15
COD (mg O_2/L)	25	100[d]	61	54	104	126	156[b]	120
Total dry weight (mg/L)	878	844	810	730	354	204	193	188
Dry weight after NaOCl (mg/L)	837	798	760	589	105	23[f]	17[f]	25[f]
Proteins in solids (mg/L)	55	58	59	158	252[e]	232[e]	129[e]	123[e]
Soluble proteins (mg/L)	0	2	3	10	17	45	62[b]	60
	Time (h)							
V6: carbon introduced with PHB, 7779 mg[a]	33	72	78	92	102	118	147	
Carbon dioxide (Σ mg)	35.2	854	1544	3109	3551	3834	3924	
Volume before sampling (L)	3.62	3.47	3.32	3.17	3.02	2.87	2.72	
Sample volume (L)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	
COD (mg O_2/L)	255[d]	43	70	72	129[b]	126	101	
Total dry weight (mg/L)	937	789	621	396	341	304	258	
Dry weight after NaOCl (mg/L)	910	666	406	132	66	58[f]	57[f]	
Proteins in solids (mg/L)	50	136	216	343[e]	346[e]	339[e]	247[e]	
Soluble proteins (mg/L)	1	9	8	21	22[b]	19	19	

[a] PHB concentration determined from sample taken before inoculation.
[b] Maximum value of COD and soluble proteins.
[c] Dilution for protein determination with water, not with NaOH.
[d] Too high COD values because of (PHB) particles.
[e] Too high protein values.
[f] PHB totally degraded, according to GC analysis.

products of degradation and metabolism, as well as proteins released into the medium. As observed from previous experiments, introduction of soluble carbon components with the inoculum (1 vol%) could be neglected. The soluble proteins were not used for calculating carbon balances, but they are included in the COD values. An increase in soluble proteins correlates with an increase in degradation activity (e.g., carbon dioxide evolution), possibly a result of the release of exoenzymes responsible for PHB degradation. The total dry weight represents the sum of biomass and residual polymer. The dry weight after NaOCl treatment corresponds to the amount of residual polymer and, along with the total dry weight, should decrease during degradation. This behavior was observed for experiments V4, V5, and V6. However, for V3 there was an apparent increase in both dry masses at the end of degradation. In this experiment the dry masses were determined by lyophilization of the samples. To avoid further experimental errors (e.g., usage of grease, usage of round bottom flasks with high weight), drying in a vacuum oven was applied later, using containers as small as possible.

Although, in all experiments, PHB was degraded totally (as indicated by GC analysis), there was always some dry mass detectable after hypochlorite treatment, possibly insoluble metabolites (e.g., polysaccharides) or unoxidized cell fragments. Especially in the degradation using a mixed culture (V6), a relatively large amount of residual material was observed.

Proteins that are included or attached to the microorganisms were used for calculation of the biomass. In some cases (especially where samples were highly diluted before protein determination), protein values were very high (see Table I). Biomass calculated from these data was higher than the total dry mass, resulting in an apparent "negative" mass of residual polymer. In such cases, the calculated residual polymer was set to zero for the calculation of the carbon balance.

Comparing the course of degradation for the four experiments, differences in the lag phases such as those observed in "Sturm test" testing [17] were observed. During V5 the degradation stirring speed was higher than in the other experiments, and as a result, degradation by *Acidovorax facilis* may have been retarded due to the higher shear stress.

The relatively long lag phase during V6 degradation was caused by the use of an inoculum (compost eluate) that was not prescreened. Nevertheless, taking into account the differences in lag phase, degradation of PHB was complete within 60 to 70 h, independent of the conditions applied or the inoculum used.

Carbon Balances

In principle, for establishing a carbon balance, three groups of components have to be considered:

(i) gaseous components,
(ii) soluble components, and
(iii) solid components.

If an aerobic process is ensured, as in the experiments described here, CO_2 is the only gaseous carbon fraction released from the microorganisms. In the case of anaerobic processes methane can be produced as well and theoretical assumptions about the relation between CO_2 and methane (e.g., Buswell equation) or a separate detection of methane (e.g., by gas chromatography) must be made. For the detection of soluble components, sum parameters like DOC (dissolved organic carbon) and COD (chemical oxygen demand) are widely applied in degradation experiments [14]. In our experiments, we chose the COD parameter because it is a relatively simple and standardized analytical method, which does not require complex and expensive analytical equipment. For the transformation of the data into the amount of carbon dissolved, we used an empirical relation [Eq. (3)] obtained from measurements with bovine serum albumin (BSA) as the soluble carbon component. From sum parameters, however, differences between possibly resistant degradation intermediates and microbial metabolic products cannot be distinguished. In the case of blends or copolymers, for instance, it could be possible, that parts of the polymer (e.g., one monomer fraction) are solubilized by the action of exoenzymes but cannot be metabolized by microorganisms. Here additional tests (HPLC or BOD measurements) are necessary to prove complete biodegradability. However, in degrading a PHB homopolymer, only one kind of monomer can be released. Thus, resistant primary degradation products are questionable. In all experiments presented here approximately 50% of the soluble carbon components could be identified as proteins.

Major experimental problems arose during detection of the solid components. The quantitative mechanical fractionation of residual polymer and biomass has been shown to be impossible because the biomass adsorbs strongly to the polymer surface and may even penetrate into the macromolecular substance (e.g., in the case of fungi). On the other hand, vigorous mechanical treatment of the samples may release small, nondegraded polymer particles from the test specimen.

Thus, indirect methods must be applied. We used two methods:

(i) determination of the biomass from protein measurements and
(ii) determination of residual polymer by oxidation of biomass with hypochlorite.

In combination with the total dry mass data, different combinations for calculating overall C balances are possible.

When determining biomass based on protein measurement, assumptions about the protein content of the biomass have to be made. An average protein content of 50% is usually reported [24], but the fraction of protein in microorganisms can vary from strain to strain and also depends on growing conditions. Protein concentrations of 55 and 65%, respectively, were determined experimentally for the strain *Acidovorax facilis* and the mixed culture used in this experiments after complete degradation of PHB. These data were used to compare carbon balances based on different assumptions. Nevertheless, exact determination of the protein content of biomass growing on a polymer will not be possible in practice without proof of the complete degradation of the polymer by independent methods.

Another assumption for establishing the carbon balances concerns the average carbon content of the biomass. For these calculations we used a value of 50% [25] carbon in biomass.

In the experiments presented here, the loss of carbon due to taking samples also has to be taken into account. The current carbon content in the fermenter was calculated from the total carbon introduced with the polymer minus the carbon released with CO_2 up to the moment of sampling.

The equations used for calculating the different components are as follows.

Carbon Dioxide.

$$c_{CO_2} = \frac{x_{CO_2} \cdot v_{air} \cdot \Delta t}{V_M} \cdot 10 \tag{1}$$

$$C_{CO_2} = \sum_{t=0}^{t} c_{CO_2}(t) \tag{2}$$

Dissolved Organic Carbon.

$$C_{COD} = \frac{COD \cdot V_F}{a \cdot M_C} \tag{3}$$

Samples.

$$C_{samples} = \sum_{t=0}^{t-1} \frac{(C_{total} - C_{CO_2}(t)) \cdot V_S(t)}{V_F \cdot} \tag{4}$$

Biomass.

$$C_{biomass} = \frac{(m_{total} - m_{NaOCl}) \cdot V_F \cdot p_{biomass} \cdot 1000}{M_C \cdot V_S} \tag{5}$$

$$C_{biomass} = \frac{c_{solidprot.} \cdot V_F \cdot p_{biomass} \cdot 1000}{x_{protein} \cdot M_C} \tag{6}$$

PHB.

$$C_{polymer} = \frac{m_{NaOCl} \cdot p_{polymer} \cdot V_F \cdot 1000}{V_S \cdot M_C} \tag{7}$$

$$C_{polymer} = \frac{(m_{total} - m_{biomass}) \cdot p_{polymer} \cdot V_F \cdot 1000}{V_s \cdot M_C} \tag{8}$$

The fraction of carbon according the total carbon introduced by the polymer is calculated from

$$F_x = \frac{C_x}{C_{total}} \cdot 100 \tag{9}$$

Using the formuls listed above, five types of carbon balances were established by combining the nine equations based on different assumptions and analytical methods. The schemes for calculating the balances are shown in Table II, where B1 to B5 indicate the different C balances and the numbers represent the different equations and assumptions. All data are related to the total

Table II. Schemes for Calculating the Carbon Balances[a]

Balance No.	Combination of components used for calculation				
	CO_2	COD	Samples	Biomass	Polymer
B1	1	+2	+3	+4	+7
B2	1	+2	+3	+5	+8
B3	1	+2	+3	+6	+9
B4	1	+2	+3	+4	+9
B5	1	+2	+3	+5	+9

[a] Component 1: From Eq. (2). Component 2: from Eq. (3). Component 3: from Eq. (4). Component 4: from Eq. (6) with the assumption of 50% protein in biomass. Component 5: from Eq. (6) with the assumption of 55% protein in biomass (V3–V5; *Acidovorax facilis*). from Eq. (6) with the assumption of 65% protein in biomass (V6; mixed culture). Component 6: from Eq. (5). Component 7: from Eq. (8) with biomass according to component 5. Component 8: from Eq. (8) with biomass according to component 6. Component 9: from Eq. (7).

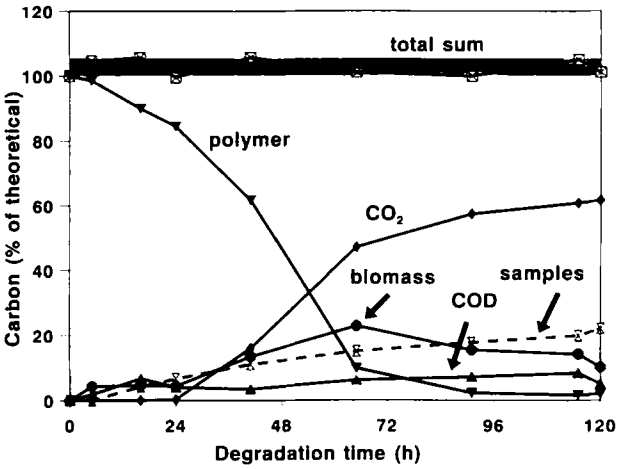

Fig. 2. Time course of PHB degradation with *Acidovorax facilis*; experiment V5; C balance B3.

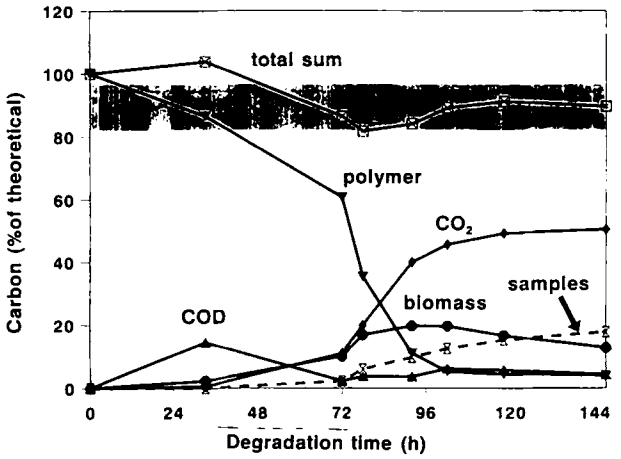

Fig. 3. Time course of PHB degradation with a compost eluate; experiment V6; C balance B3.

amount of carbon introduced into the system as PHB. As an example the fractions of carbon for experiments V5 (*Acidovorax facilis*) and V6 (compost eluate) based on calculation scheme B3 are plotted against degradation time in Figs. 2 and 3, respectively.

PHB degradation in these experiments demonstrates the behavior of a conventional batch fermentation. After a lag phase, logarithmic growth of the microorganisms can be observed, with a microbial activity characterized by carbon dioxide evolution. As expected, the PHB concentration decreases during the log phase of microbial growth. After the polymeric carbon is consumed, CO_2 evolution levels to a plateau region. During this phase, biomass decreases, indicating partial lysis and metabolization of the cells. Note that in experiment V5, the fraction of carbon released as carbon dioxide only minimally exceeds the 60% level required by the OECD for a readily biodegradable substance [15], yet the C-balance data indicated that the PHB had been degraded totally. In the case of degradation with the mixed culture, only 50% of CO_2 was released at this point. It was determined by GC analysis that the dry mass after hypochlorite treatment (i.e., residual "polymer") at the end of the experiments was not PHB but probably consisted of insoluble metabolites (e.g., polysaccharides) or cell fragments not destroyed by the chemical treatment. Determining the residual polymer from protein measurements of the total dry mass, after 65 h (V5) and 92 h (V6), respectively, the calculated biomass was greater than the total dry mass. Thus, the polymer concentration was considered to be zero.

The fraction of carbon, derived from the COD value of the soluble materials, is in the region of about 4 to 8% and no significant increase could by observed during the degradation experiments. The relatively high COD values at the beginning of the experiment were not

Table III. Average Data of Total Carbon Detected for Different Calculation Schemes of Degradation Experiments V3–V6

	Total carbon detected (% of theory): calculation scheme for balance				
Expt. No.	B1	B2	B3	B4	B5
V3[a]	93.5 (±14.5)	93.3 (±15.5)	91.9 (±15.2)	84.7 (±9.4)	82.1 (9.9)
V4[a]	105.9 (±8.0)	102.8 (±5.0)	101.2 (±2.7)	108.7 (±10.3)	103.7 (7.0)
V5[a]	107.7 (±7.3)	104.4 (±4.0)	102.8 (±2.6)	114.9 (±7.2)	109.5 (4.7)
V6[a]	100.9 (±13.6)	98.9 (±11.6)	89.4 (±7.2)	110.1 (±8.9)	106.9 (8.2)
Average[b]	104.9 (±10.0)	102.0 (±7.8)	97.9 (±7.6)	111.5 (±8.7)	107.0 (±6.7)

[a] Average of all data from one degradation experiment.
[b] Average of all data from experiments V4, V5, and V6.

caused by the addition of external carbon from the inoculum but may reflect the fact that in the initial phase of the experiments the dry mass consists mainly of PHB that does not yield a stable pellet after centrifugation; consequently, the supernatant may contain some PHB particles.

The carbon balances based on the different methods were scattered around a value close to 100%. In Table III, the average of the total carbon calculated for the different balancing schemes (at completion of three experiments) is presented for the different experiments. Average values for all data for each balance also are listed. Experiment V3 was not been included in this calculation, because the analytical methods still were under development.

DISCUSSION

The aim of this investigation was to develop improved testing of the biodegradability of polymers by means of establishing carbon balances under conditions comparable to standard degradation experiments. Our data confirm that, as suggested by other investigations [17], the amount of CO_2 evolved during degradation is not a secure measure of the total extent of degradation for polymers. In all experiments, the carbon dioxide level was in the range of only 40 to 50%, while the residual polymer concentration was almost zero. Further release of CO_2 was caused by the degradation of biomass.

According to OECD guidelines, readily biodegradable materials must show a CO_2 evolution of more than 60%; thus, PHB would not meet this requirements. Nevertheless, carbon dioxide is a major component of the carbon balance and there is a variety of methods for accurately determining this carbon fraction.

Standard methods also can be used for the registration of determine the soluble carbon fraction. In our investigations we chose the determination of chemical oxygen demand as the standard method. The empirical assumption about the relation between COD and the carbon content, which we derived in experiments with BSA, qualifies this method to some extent. If the fraction of soluble carbon components is relatively low, as observed for the biological degradation of PHB, only a small error due to this assumption can be expected. Determining the dissolved carbon as DOC with an appropriate analytical method would avoid this error, but requires additional analytical equipment. Both methods measure sum parameters, thus it not possible to distinguish if the carbon components detected are microbial metabolic products (e.g., proteins) or if nondegradable intermediates (e.g., from copolymers) are present. In the case of higher amounts of soluble carbon components, further degradability could be investigated by BOD measurements; alternatively, intermediates could be identified by other methods (e.g., chromatography).

The main problem in establishing a detailed carbon balance is associated with nonsoluble materials. Selective and quantitative methods for determining residual polymer (e.g., by chromatography) are not applicable for all polymers. Calculating biomass from indirect methods (e.g., the amount of proteins) is a common practice but requires several assumptions (e.g., on the content of proteins in biomass and the carbon content of biomass). The use of an analytically determined protein content of the specific microorganisms used in the degradation experiment instead of the average value reported in the literature, i.e., 50%, significantly enhances the accuracy of the carbon balances calculations but would be somewhat impractical for standardized testing. When the microorganisms are grown on the polymer itself, complete degradation of the plastic must be ensured before determining the protein content of the biomass, but obtaining information about the degree of degradation is the goal of the test itself. Protein contents from microorganisms cultivated on other media cannot be used, because protein concentration in the biomass is depending on growing conditions.

In principle, the unreasonable high protein values, determined in some cases, give evidence of insufficient accuracy of the protein detection method applied. Alternative methods for the determination of biomass, e.g., based on ATP or DNA measurements, should be tested, but they have principally no advantage from the point of view of the assumptions which have to be made.

In contrast, calculating biomass from the mass difference before and after treatment of the dry mass with a hypochlorite solution does not include any assumption. If it has been ensured that NaOCl treatment does not affect the polymer mass and if the analytical procedure is optimized with regard to the handling of small masses, good results can be obtained. Analysis of the residual material with TOC methods may improve the accuracy of the carbon balance. As observed from the degradation experiment with the mixed culture from compost eluate, there may be some problems with water-insoluble metabolic products or cellular material that is not destroyed by hypochlorite.

Despite all of the assumptions and problems, the average total sums of all components from the respective balancing schemes B1, B2, and B3 were in the range of 89 to 108%. When protein measurements as well as

data from the hypochlorite method were used for calculating the balances, deviations from the theoretical value of 100% were large. The best method, with respect to the total sum of carbon detected and a small standard deviation, was the calculation based on the hypochlorite treatment, although there is some evidence that problems may arise from mixed cultures. Determination of biomass from protein measurements in combination with the experimentally determined protein content of the biomass gives comparable results. However, for the reasons mentioned above, this method is impractical for standardized testing.

In conclusion, our investigations have shown that it is possible to obtain satisfactory carbon balances with the hypochlorite method applied, provided that this procedure is optimized for the test conditions and adapted for the test material under investigation.

Calculating carbon balances would improve the reliability of biodegradation test methods and should be introduced in standard tests. The total sum of carbon detected can be used as one parameter to validate the test. The degradation rate can be determined as usual by monitoring the microbial activity (e.g., determination of CO_2), but the degree of degradation at the end of the test should be specified from one carbon balance as the sum of carbon from CO_2 and biomas. To establish one C balance at the end of the test would be acceptable under economical aspects even for standard test procedures; test times may be shortened, because it would not be necessary to run the degradation over a fixed time period. The test could be terminated when the CO_2 evolution starts to level to a plateau.

Nevertheless, despite the good results of the calculations presented here, alternative analytical methods may further enhance the accuracy of carbon balancing in such systems for improved evaluation of the biodegradability of polymers.

NOMENCLATURE

$\Delta t(h)$	Time interval for determining average CO_2 concentration
a (mg_{O_2}/mg_C)	Empirical factor (=3)
c_{CO_2} (%)	Average concentration of carbon dioxide in the time interval Δt
$C_{biomass}$ (mmol)	Carbon contained in biomass
C_{CO_2} (mmol)	Carbon contained in carbon dioxide
C_{COD} (mmol)	Carbon contained in soluble products
COD (mg O_2/L)	Chemical oxygen demand
$C_{polymer}$ (mmol)	Carbon contained in residual polymer
C_{sample} (mmol)	Carbon contained in samples taken from system
$c_{solidprot.}$ (g/L)	Concentration of proteins from biomass
C_{total} (mmol)	Total carbon introduced by the polymer
C_x	Carbon contained in fraction X of the balance
F_x (%)	Fraction of component x in the carbon balance
$m_{biomass}$ (g)	Dry cell mass
M_C	Molar mass of carbon (=12.01)
m_{NaOCl} (g)	Dry mass after NaOCl treatment
m_{total} (g)	Total dry mass
$p_{biomass}$ ($mg_C/mg_{biomass}$)	Carbon content of the biomass
$p_{polymer}$ ($mg_C/mg_{polymer}$)	Carbon content of the polymer
t	Time of sampling
v_{air} (L/h)	Air flow
V_F (L)	Volume of the medium at time t
V_M (L/mol)	Molar gas volume
V_S (L)	Volume of the sample taken from the medium
x_{CO} (%)	Fraction of CO_2 in exhaust air
$x_{protein}$ ($mg_{protein}/mg_{biomass}$)	Fraction of protein in biomass

REFERENCES

1. R. Narayan and S. Blomembergen (1991) *J. Am. Chem. Soc. Polym. Preprints* **32**(2), 119–120.
2. M. M. Nir, J. Miltz, and A. Ram (1993) *Plastics Eng.* **3.93,** 75–93.
3. Y. Doi (1990) *Microbial Polyesters*, VCH, New York.
4. G. A. Birrer, A.-M. Cromwick, S. P. McCarthy, and R. A. Gross (1992) *PMSE* **67,** 134–136.
5. D. L. Kaplan, J. Mayer, S. Lombardi, B. Wiley, and S. Arcidiacono (1989) *J. Am. Chem. Soc., Polym. Preprints* **30**(1), 509–510.
6. J. E. Glass and G. Swift (1990) *Agricultural and Synthetic Polymers—Biodegradability and Utilization*, ACS Symposium Series 433, Washington, DC.

7. K. Sakai, N. Hamada, and Y. Watanabe (1986) *Agr. Biol. Chem.* **50**(4), 989–996.
8. A. Tsuchii and K. Takeda (1990) *Appl. Environ. Mikrobiol.* **56**(1), 269–274.
9. Y. Sirakura, T. Fukui, T. Saito, Y. Okamoto, T. Narikawa, K. Koide, K. Tomita, T. Takamasa, and S. Masamune (1986) *Biophys. Acta* **880**, 46–53.
10. H. Bauer and A. J. Owen (1988) *Colloid Polym. Sci* **266**(3), 241–247.
11. U. Witt, R.-J. Müler, J. Augusta, H. Widdecke, and W.-D. Deckwer (1994) *Macromol. Chem. Phys.* **195**, 793–802.
12. M. Parikh, R. A. Gross, and S. P. McCarthy (1992) *PMSE* **66**, 408–410.
13. J. Augusta, R.-J. Müller, and H. Widdecke (1992) *Chem.-Ing.-Tech.* **64**(5), 410–415.
14. R. Wagner (1988) *Methoden zur Prüfung der Biochemischen Abbaubarkeit Chemischer Substanzen*, VCH Verlagsgesellschaft, New York.
15. OECD Guidelines for Testing of Chemicals (1992) 301 B, *Ready Biodegradability*.
16. ASTM 5209-91 (1991) *Standard Test Method for Determining the Aerobic Biodegradation of Plastic Materials in the Presence of Municipal Sewage Sludge*.
17. R.-J. Müller, J. Augusta, and M. Pantke (1992) *Mater. Organismen* **27**, 179–189.
18. J. Augusta, R.-J. Müller, and H. Widdecke (1993) *Appl. Microbiol. Biotechnol.* **39**, 673–678.
19. D. H. Williamson and J. F. Wilkinson (1958) *J. Gen. Microbiol.* **19**, 198–209.
20. O. W. Lowry (1951) *J. Biol. Chem.* **193**, 265–275.
21. A. Sperandio and P. Püchner (1993) *Wasser Abwasser* **134**(8), 482–485.
22. H. Brandl, R. A. Gross, R. W. Lenz, and C. Fuller (1988) *Appl. Environ. Microbiol.* **54**, 1977–1982.
23. G. Braunegg, B. Sonnleitner, and R. M. Lafferty (1978) *Eur. J. Appl. Microbiol. Biotechnol.* **6**, 29–37.
24. H. G. Schlegel (1985) *Allgemeine Mikrobiologie*, Thieme Verlag, Stuttgart.
25. H. W. Dellweg (1987) *Biotechnologie-Grundlagen und Verfahren*, VCH Verlag, New York.