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Anaerobic degradation of poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-3-hydroxyvalerate

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Abstract

The anaerobic degradation of the polyesters poly-3-hydroxybutyrate (PHB) and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) was investigated with special regard to intermediate products, kinetics, and yields. During the degradation of PHBV acetate, propionate, n-butyrate, and n-valerate were detected. Additionally, 3-hydroxybutyrate and 3-hydroxyvalerate and four dimeric esters of these two molecules were identified by GC-MS measurements. Three different test systems for the anaerobic degradation of polyesters were studied. It was not possible to get reproducible results by means of the Anaerobic Sturm-test, a simple system based on carbon dioxide measurement. Secondly, a system based on the GC measurement of accumulated organic acids was investigated. A degradation of 90% in two days was calculated by a carbon balance. Best results were reached with the third test system based on the measurement of methane with a gas meter. A degradation of 99% was observed within 30 days.

Abbreviations: Ac – Acetate; ASTM – American Society for Testing and Materials; CEN – Comité Européenne de Normalisation; COD – Chemical Oxygen Demand; DIN – Deutsche Industrie-Norm; HV – Hydroxyvalerate; ISO – International Organization for Standardization; n-But – n-Butyrate; n-Val – n-Valerate; PHB – Poly-3-hydroxybutyrate; PHBV – Poly-3-hydroxybutyrate-co-3-hydroxyvalerate; PHV – Poly-3-hydroxyvalerate; Prop – Propionate; TOC – Total Organic Carbon; VSS – Volatile suspended solids

Introduction

Up to now there exists no generally accepted definition of biodegradability and biodegradation. The definition of DIN is the most stringent one among the definitions of ASTM, CEN, ISO and DIN. According to this definition the biodegradation of a plastic material is a process which leads to a change of its chemical structure and is caused by biological activity. The endproducts are naturally occurring metabolites (DIN FNK 103.2). However, accurate test methods to analyse kinetics and the conversion of polymers to metabolic products are not yet available.

Most methods of the investigation of anaerobic degradation of low molecular substances are based on volumetric and manometric techniques (ASTM E 1196-1987, Baumann & Schefer 1990, Birch et al. 1989, and Fedorak & Hrudey 1983). They involve the measurement of methane and carbon dioxide production which are the final products of anaerobic biodegradation. The comprehensive work of Shelton and Tiedje (1984) reports a method using a gastight syringe and a pressure transducer to monitor gas production. A mixture of anaerobic sludge diluted in a mineral salts medium and a suitable quantity of test chemical was digested in a sealed vessel. The net gas production was measured by the pressure in the headspace above the digesting liquid.

Only few authors reported suitable test methods of anaerobic degradation of polymers. The evaluation of

polymer degradation in a controlled microbial chemostat was reported by Loomis et al. (1991). Changes in physical properties (tensile strength, elongation) and molecular weight distribution were used as degradation parameters. McCartin et al. (1990) described a simulated landfill study on the accelerated biodegradability of plastic materials. A test method to determine the anaerobic biodegradation of plastic materials in the presence of municipal sewage sludge was published by the American Society for Testing and Materials (ASTM D 5210-1992). Some principle considerations about the microbiological procedures, analytical techniques, and requirements for polymer degradation tests were described by Augusta et al. (1992).

Poly-3-hydroxybutyrate (PHB) and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) were shown to be biodegradable and biocompatible plastics (Holmes 1985) and consequently seem to be suitable as test materials for the development of biodegradation test systems. The anaerobic degradation of exogenous PHB by members of the genus *Pseudomonas* was reported (Chowdhury 1963) and the enzymology was investigated (Lusty & Doudoroff 1966). The immediate hydrolysis product is usually D-3-hydroxybutyric acid (Chowdhury 1963). Anaerobic degradation of exogenous PHB was reported to occur in sewage sludge and in bovine serum (Holmes 1985). Janssen and Harfoot (1990) reported the isolation and characterization of the PHB-degrading obligatory anaerobic bacterium *Ilyobacter delafieldii*. The 3-hydroxybutyrate, formed during growth on PHB, was fermented to acetate, butyrate, and H_2. The results of these experiments suggested the production of an extracellular PHB depolymerase. Budwill et al. (1992) described the fermentation of poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-3-hyroxyvalerate to methane and carbon dioxide within 16 days by an anaerobic sewage sludge consortium. The transformation of substrate carbon to biogas was reported to be 87% for PHB and 96% for PHBV with 13% HV. On the whole, the measurement of biogas production seems to be appropriate to investigate the anaerobic degradation potential of polymers.

This communication gives data about the anaerobic degradation of the polyesters PHB and PHBV with special regard to intermediate products, kinetics, and yields as well as information on analytical methods. Some difficulties and limitations of anaerobic degradation of the polyesters were identified and three experimental arrangements as a basis for the development of tests for anaerobic degradation of polymers are suggested. Firstly, a system derived from the Aerobic Sturm-test based on the measurement of carbon dioxide evolution was investigated. The Sturm-test (Sturm 1973) and the Modified Sturm-test (approved by the OECD in 1981) are appropriate for the evaluation of the aerobic degradability of low molecular weight organic substances by the determination of the carbon dioxide produced in this microbial process. With regard to the degradation of polymers, some further modifications of the Sturm-test were performed in several laboratories by the International Biodeterioration Research Group (IBRG) (Müller et al. 1992). Secondly, a system based on the measurement of accumulated organic acids was used. To avoid inaccuracies due to the solubility of carbon dioxide in the digesting liquor, experiments based on methane measurement only are given as a third possibility.

Materials and methods

Polymers

The polyesters PHB and PHBV were given by ICI Bio Products & Fine Chemicals, Deutsche ICI GmbH, Frankfurt/Main, Germany. PHB powder: surface area 1.39 $m^2\ cm^{-3}$ and mean particle size 9.8 μm by laser spectrometry. PHBV (8.4 mol% HV) powder: molecular weight $1.1 \cdot 10^6$ g mol^{-1} by gel permeations chromatography, surface area 0.27 $m^2\ cm^{-3}$ and mean particle size 46.4 μm by laser spectrometry. PHBV (19.1 mol% HV) powder: molecular weight $7.2 \cdot 10^5$ g mol^{-1} by intrinsic viscosity, surface area 0.35 $m^2\ cm^{-3}$ by laser spectrometry and 2.3 $m^2\ g^{-1}$ by nitrogen sorption, mean particle size 42.1 μm by laser spectrometry and 57 μm by suspension cell light scattering.

Inoculation

Methanogenic sludge

For inoculation of the Anaerobic Sturm-test and methane production experiments anaerobic sludge of a waste water plant of a sugar factory was used. Prior to inoculation the sludge was concentrated by centrifugation at 5–10 °C. The inoculum contained 64% (w/w) total dry-mass. Further analysis of this dry-mass showed that the main part consisted of 92% (w/w) $CaCO_3$ and that only 4% (w/w) were VSS.

Selective culture

The inoculation of the experiments with organic acid accumulation was carried out with selective cultures, which were enriched on a medium containing polymer powder as the sole carbon source. After sparging nitrogen into 100 ml of a mineral medium containing 100 mg polymer powder, 1 g methanogenic sludge (wet weight) was used for inoculation. This mixture was incubated at 35 °C and pH 7.0-7.6 for eight days in 500 ml erlenmeyer flasks under anaerobic conditions.

Medium

The mineral medium for degradation tests in liquid cultures contained per litre 258 mg urea (Riedel de Haën, Seelze, Germany), 360 mg $mgCl_2 \cdot 6\ H_2O$, 60 mg yeast extract (Merck, Darmstadt, Germany), 1.40 mg $FeCl_2 \cdot 4\ H_2O$, 0.27 mg $NiCl_2 \cdot 6\ H_2O$, 0.95 mg $MnCl_2 \cdot 4\ H_2O$, 0.34 mg $CoCl_2 \cdot 6\ H_2O$, 0.064 mg $CuSO_4$, 0.36 mg $ZnSO_4 \cdot 7\ H_2O$, 0.036 mg $KAl(SO_4)_2 \cdot 7\ H_2O$, 0.02 mg H_3BO_3, 0.04 mg $Na_2SeO_3 \cdot 5\ H_2O$, 0.04 mg $Na_2WO_4 \cdot 2\ H_2O$, 0.04 mg $Na_2MoO_4 \cdot 2\ H_2O$, 67 mg $Na_2HPO_4 \cdot 12\ H_2O$, 29 mg KH_2PO_4.

The pH of the mineral medium was adjusted to 7.2 with H_3PO_4. During degradation experiments in which methane production was measured, 0.5 g $Na_2S \cdot \times H_2O$ (60–62%) and 0.5 g cysteine hydrochloride were supplied to the mineral medium to decrease the redox potential. In this mineral medium the pH was adjusted to 7.2 using 1 M NaOH.

Analytical methods

Volatile fatty acids were measured using a gas chromatograph with a flame ionization detector (Shimadzu, GC-14A, Tokyo, Japan) equipped with a 1.6 m glass column and packed with Chromosorb 101 (mesh 80/100) (Manville, Denver, USA). The carrier gas N_2 was adjusted to a flow rate of 40 ml min^{-1}. The column temperature was set to 225 °C and the injector temperature was 250 °C.

The intermediates 3-hydroxybutyrate, 3-hydroxyvalerate and the dimeric esters of 3-hydroxybutyrate and 3-hydroxyvalerate were analysed at the Chemical Institute of the Technical University of Braunschweig, Germany, by GC-MS after derivatization with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA). A mass spectrometer Finnigan MAT 4515 (Finnigan MAT, Bremen, Germany) with electron-impact ionization and a gas chromatograph Carlo Erba HRGC 5160 (Carlo Erba, Milan, Italy) equipped with a 30 m $\times$ 0.32 mm quartz capillary column with a phase of methylsilicone were used. The column temperature was set to 70–300 °C (6 °C min^{-1}).

The content of carbon dioxide and methane in the biogas was determined by gas chromatography (Shimadzu GC-14A) equipped with a thermal conductivity detector and two parallel stainless steel columns (3 m $\times$ 1/8″) packed with Porapak Q (mesh 80/100) and molecular sieve 5A (mesh 60/80). Helium as the carrier gas was adjusted to a flow rate of 30 ml min^{-1}. The column temperature was 35 °C, the injector temperature 100 °C and the detector temperature 120 °C. Gas samples of the headspace gas were taken using a gastight syringe.

Volatile suspended solids (VSS) were determined as the difference between total dry mass and ash after drying at 105 °C and 550 °C.

Protein determination was carried out with the method described by Bradford (1976) after treating the sample with 1 N NaOH (Herbert et al. 1971).

Theoretical amounts of gas production

The theoretical amounts of gas production from the degradation of definite amounts of polymers were calculated on the basis of the Buswell-equation (Buswell & Mueller 1952):

$$C_nH_aO_b + (n - a/4 - b/2)H_2O \rightarrow$$

$$(n/2 - a/8 + b/4)CO_2 + (n/2 + a/8 - b/4)CH_4$$

The maximum gas amounts during the degradation process of PHB and PHV are given by the following equations:

PHB
–(O–C(CH_3)H–CH_2–C(O))– + 1.5 H_2O
$\rightarrow$ 1.75 CO_2 + 2.25 CH_4
PHV
–(O–C(CH_2–CH_3)H–CH_2–C(O))–
+2 $H_2O \rightarrow$ 2 CO_2 + 3 CH_4

Thus, for the degradation of the copolymer PHBV with 19.1% Hydroxyvalerate [–(O–C(CH_3)H–CH_2–C(O))$_n$–(O–C(CH_2–CH_3)H–CH_2–C(O))$_m$–; n = 0.809, m = 0.191] maximal amounts of 1.8 mol CO_2 and 2.39 mol CH_4 were calculated. For PHBV with 8.4% HV the values are 1.77 mol CO_2 and 2.31 mol CH_4.

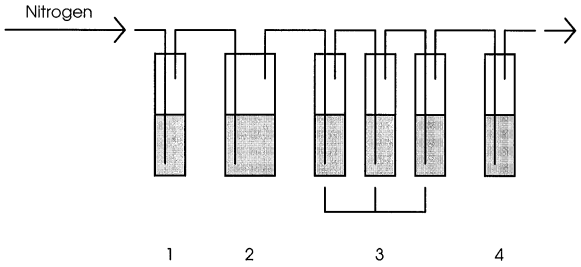

Figure 1. Scheme of degradation test apparatus with carbon dioxide measuring (Anaerobic Sturm-test).

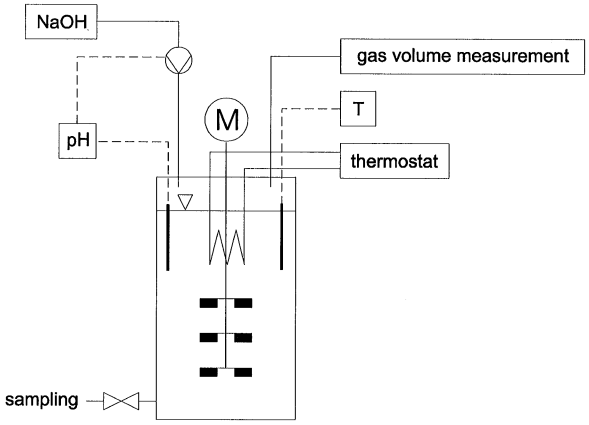

Figure 2. 6 l stirred tank reactor with pH control, temperature control, and gas metering for the fermentations with quantitative biogas determination(T: thermometer, M: stirring motor).

Degradation tests

Anaerobic Sturm-test

The Anaerobic Sturm-test was based on the measurement of the carbon dioxide evolved during biological degradation. The test apparatus (Figure 1) consisted of four basic units and was gassed continuously with 1.2 l h^{-1} nitrogen. The gas stream was pre-treated with 100 ml 5 m KOH to remove carbon dioxide (1). In the 500 ml degradation vessel (2) 0.1% (w/w) polymer powder was present and 1% (w/w) methanogenic sludge was added as inoculum (0.025% VSS). The liquor was mixed by a magnetic stirrer in the dark at 35 °C. Three gas washing bottles (3), each filled with 100 ml 0.025 N $Ba(OH)_2$, were used for absorption of carbon dioxide. Another bottle (4) filled with 100 ml 5 m KOH prevented back flow of atmospheric carbon dioxide. The connecting tubes consisted of polyvinylchloride. For all solutions carbon dioxide free distilled water was used. For each degradation test a parallel blank test was performed to calculate the amount of carbon dioxide evolved by the inoculum. These parallel tests were done with the same amount of inoculum but without polymer material. The relatively small amount of sludge was used to minimise the amount of carbon dioxide evolution from the inoculum.

For carbon dioxide determinations, the $Ba(OH)_2$ solutions with precipitated carbonate were titrated with 0.05 m HCl. Prior to titration the precipitate was removed by filtration.

Degradation tests with acid accumulation

0.1% (w/w) PHBV powder containing 8.4% HV was fermented by a 2% (v/v) selective culture in the mineral salts medium at 35 °C and pH 7.2 in the dark. The sludge content of the culture liquor was below the detection limit. The experimental set-up and the carbon dioxide determination were equivalent to the procedures described for the Anaerobic Sturm-test. However, the Sturm-test degradation vessel was replaced by a 500 ml stirred reactor with pH control and temperature control. Samples were taken by a syringe. In contrast to the Sturm-test procedure the reactor containing the test solution was gassed only for one hour with nitrogen to remove dissolved carbon dioxide before starting the experiments. At the end of the experiment the whole system was gassed again for one hour to carry produced carbon dioxide to the absorption flasks filled with $Ba(OH)_2$.

As a result of using a selective culture for inoculation the lime content was extremely low and consequently carbon dioxide production based on the $CaCO_3$ content was negligible. The amount of organic acids added with the inoculum was also negligible. Thus, a blank test was not necessary.

Degradation tests with methane determination

The experimental set-up of the fermentations with quantitative biogas determination consists of a 6 l stirred reactor equipped with pH control, temperature control, and gas metering (Figure 2). The experiments were carried out with 10% (w/w) methanogenic sludge (0.25% VSS) dissolved in mineral salts solution and 1% (w/w) polymer powder at 35 °C and pH 7.2 in the dark. Oxygen was removed by sparging the test solution with nitrogen for 30 minutes at the beginning of the experiments.

To avoid measurement of additional gas production caused by digestible organic materials in the inoculum, the complete test liquor (mineral medium and sludge)

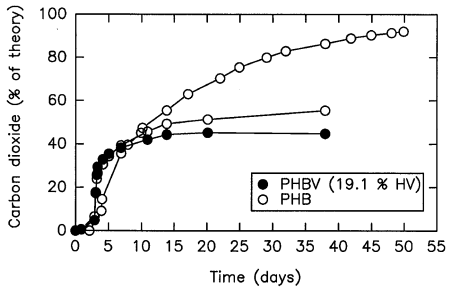

Figure 3. Carbon dioxide production observed during three degradation experiments with 0.1% PHB and 0.1% PHBV (19.1% HV) using a modified Anaerobic Sturm-test as degradation test apparatus (0.025% VSS).

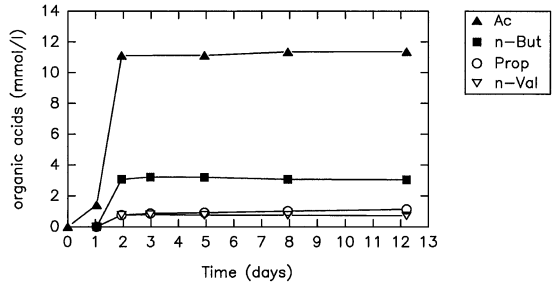

Figure 4. Accumulation of organic acids by a selective culture during fermentation of 0.1% PHBV (8.4% HV) at 35 °C and pH 7.2.

was incubated for several days without polymer until no gas evolution was observed.

The amount of gas production was determined by using a gas meter (minimum gas flow 0.2 l h^{-1}, Ritter, Bochum, Germany). The methane content of the biogas was determined by gas chromatography as described above. The theoretical quantity of methane produced under anaerobic conditions was calculated on the basis of the Buswell-equation. Gas formation was determined on basis of the mol volume of ideal gases at appropriate pressure and temperature.

Results and discussion

Degradation tests with carbon dioxide determination (Anaerobic Sturm-test)

The intention of the experiments described in this communication was to study the possibility of determining the anaerobic degradation of polyhydroxyalkanoates in a simple test system derived from the Sturm-test. The decisive modification was the use of nitrogen as carrier gas instead of compressed air for CO_2 transport to the $Ba(OH)_2$ flasks.

A number of ten experiments were carried out with this test system. Complete degradation of PHB and PHBV was observed in two runs only. For the other experiments the degradation was determined to be 39–55% within ten days. Figure 3 shows the evolution of carbon dioxide during three typical degradation experiments. The carbon dioxide evolution of simultaneous blank tests was subtracted. One test had nearly complete and two tests only partial degradation. The degradation curves show a sigmoid shape with lag phases ranging from one to two days. Nearly all curves show a plateau after ten days. An average increase of 19 mg l^{-1} protein was measured. Under the assumption that biomass contains 50% of protein, a polymer-carbon conversion to biomass of 6% was calculated. The evolution of carbon dioxide from a test substrate represents a direct parameter of mineralization (Wagner 1988). Although CO_2 measurement is an appropriate method to assess complete biodegradation of a material and to obtain information about the kinetics of the process, only further measurements will give sufficient information to establish a carbon balance in case of incomplete degradation.

In order to explain the incomplete mineralization, experiments with a degradation vessel which allowed to take samples during the process were performed. In fact, accumulation of the organic acids acetate, n-butyrate and i-butyrate during PHB degradation and in addition to that propionate and n-valerate in the case of PHBV degradation was observed. The reasons for the disturbed mineralization are not clear. One possibility is that traces of oxygen in the nitrogen stream disturbed the methanogenis which resulted in accumulation. The negative effect of constant CO_2 stripping should also be taken into consideration (S. Schoberth, pers. comm.).

Because of the contradictory results the Anaerobic Sturm-test remains questionable as an experimental procedure for testing biodegradation of polymers.

Degradation tests with accumulation of volatile organic acids

When using a selective culture as inoculum, the mineralization of 0.1% (w/w) PHBV (8.4% HV) was incomplete. Figure 4 shows the accumulation of volatile organic acids during the degradation process. After two days the acid concentrations reached a constant level of 11.24 mmol l^{-1} acetate, 1.0 mmol l^{-1} propionate, 3.0 mmol l^{-1} n-butyrate, and 0.7 mmol l^{-1}

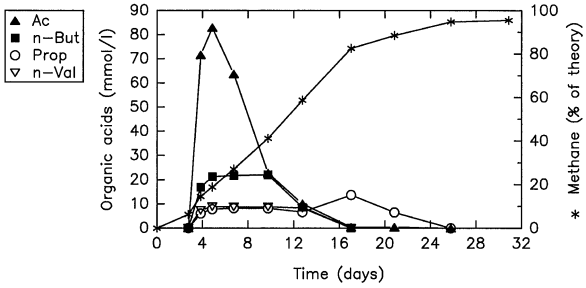

Figure 5. Methane production (∗) and formation of organic acids by an anaerobic sludge culture (0.25% VSS) during fermentation of 1% PHBV (8.4% HV) at 35 °C and pH 7.2.

n-valerate. This corresponds to a conversion of polymer carbon to organic acids of 87%. With a conversion of 2% polymer-carbon to carbon dioxide and 1% to biomass, a total degradation of 90% in two days was reached. These results and the fact that no methane could be detected in the headspace gas indicated that fermentative bacteria were the only active species during the degradation process under the test conditions described. This can be explained by the high contents of substrate (polymer) compared to the bacterial mass. Acetogenic and methanogenic bacteria were obviously inhibited by the formed organic acids. As no oxygen in the headspace of the reactor could be detected by GC a disturbance of acetogenic and methanogenic bacteria by oxygen is unlikely.

As the polymers were transformed to naturally occurring metabolites which are known to be degradable, this principle can also be used for the evaluation of biological degradability. An advantage of this system is that only few milligrams of test material are necessary. However, a standardisation of such a test method is difficult since the metabolic products of new polymers are not known. The analytical requirements are very high.

Degradation tests with methane determination

PHBV (8.4% HV) was fermented to carbon dioxide and methane within 30 days by an anaerobic sludge culture (Figure 5). For the experiments with 1% (w/w) polymer and 10% (w/w) methanogenic sludge a conversion of 95% to biogas was calculated on the basis of the amount of methane produced during the degradation process. Assuming 4% polymer-carbon conversion to biomass (calculated from protein content) a total degradation of 99% was reached in 30 days. After three days organic acids were formed as intermediates and acetate, propionate, n-butyrate and n-valerate were detected. After five days of incubation, 67% of the polymer-carbon were converted to organic acids: 82.8 mmol l^{-1} acetate, 7.8 mmol l^{-1} propionate, 21.2 mmol l^{-1} n-butyrate and 8.9 mmol l^{-1} n-valerate. These results were reproduced in further experiments.

In another equivalent degradation experiment with the polyester PHBV the intermediate hydrolysis products 3-hydroxybutyrate, 3-hydroxyvalerate and four dimeric esters of these two molecules were detected:

$HOCH(CH_3)CH_2C(O)OCH(CH_3)CH_2C(O)OH$
$HOCH(C_2H_5)CH_2C(O)OCH(C_2H_5)CH_2C(O)OH$
$HOCH(CH_3)CH_2C(O)OCH(C_2H_5)CH_2C(O)OH$
$HOCH(C_2H_5)CH_2C(O)OCH(CH_3)CH_2C(O)OH$

In addition, experiments with only 0.1% PHBV and 1% methanogenic sludge (0.025% VSS) and gas measurement by a fluid displacement system were carried out. However, the determination of the degradation levels led to values of 77–86% in 16 days. Gas chromatographic analysis of the supernatants showed that no organic acids were accumulated at the end of all experiments. The results indicate, that this gas measurement method is not suitable for the detection of such low amounts of biogas. As the theoretical biogas evolution during the reaction is about 6 l, small leaks in the apparatus lead to significant errors in the carbon balance.

In all, the system based on methane production measurements was found to be an appropriate method to test the anaerobic degradation potential of polymers. However, the requirement for obtaining satisfactory results is a sufficient and well detectable gas production rate. The quantitative and qualitative analysis of degradation intermediates, e.g. by GC or HPLC, are useful to get further information about the degradation process. In case of insoluble polymers as test materials, the chemical oxygen demand (COD) or total organic carbon (TOC) will give further information about the quantity of intermediates and the degree of mineralization.

Conclusions

It was tried to adapt the application of the Sturm-test for anaerobic digestion. With the determination of CO_2 in analogy to the Sturm-test, partly insufficient results were obtained. As discussed above traces of oxygen might be the reason for the incomplete anaerobic degra-

dation. It should also be taken into consideration that constant stripping of CO_2 might generally disturb the methanogenis. Thus, this simple experimental set-up is not adequate for the investigation of anaerobic degradation.

The degradation tests with accumulation of volatile organic acids showed that the use of selective cultures without sludge leads to incomplete methanization. As the polymers were transformed to naturally occurring metabolites which are known to be degradable, this principle can also be used for the evaluation of biological degradability.

Because of the described disadvantages of the Anaerobic Sturm-test the determination of methane as a final product of anaerobic polyester degradation is considered to be a more appropriate basis for the quantitative evaluation of biodegradability. It is essential to apply an appropriate inoculum with sufficient diversity of microorganisms under experimental conditions. It must be verified that the conversion is complete, i.e. that no volatile fatty acids remain in solution due to inhibition phenomena. It is also adequate to analyse fatty acids as intermediates and to draw up a mass balance of degradation of these intermediates, which however requires more analytical equipment.

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