# The Determination of Anaerobic Biodegradability of Pharmaceutical Waste Using Advanced Bioassay Technique

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The success of the newly developed methodology to evaluate anaerobic biodegradability of organic compounds using the Micro Oxymax respirometer and TOC analyzer made this method potentially useful for a variety of investigations. Regarding the many parameters measured during the experiment in the liquid and gas phases, the method provides good insight into the kinetics of anaerobic process and accurate mass balance, which could be obtained at any time during the assay. This method was used for determining of anaerobic biodegradability of the waste from the pharmaceutical industry-waste fermentation broth, and for the proposal of operational parameters for the start-up of the continuous process. The tests were performed in 250 ml vials at a wide range of initial substrate loading ratios (0.05-4.6 $g_{TOC}\ g_{VSS}^{-1}$). The average biodegradability of the substrate was 92 % at initial loading ratios between 0.05 and 1.7 $g_{TOC}\ g_{VSS}^{-1}$. At higher initial loading ratios 2,3 $g_{TOC}\ g_{VSS}^{-1}$ and more, the inhibition of anaerobic biodegradation was detected. Retention times needed for a 70 % and 85 % degradation efficiency were evaluated and used as a proposal for the starting-up substrate loading rate of the continuous reactor operation. An optimum initial substrate mass concentration without an inhibition effect was estimated and the fraction of methane gas in produced biogas was calculated for each initial loading.

*Key words:*
Bioassay test, wastewater treatment, operational parameters, anaerobic biodegradability, TOC

## Introduction

The involvement of new technologies and products in the pharmaceutical industry causes some problems with the disposal of highly concentrated wastes. In such a case the anaerobic pretreatment seems to be an advisable technology. The selection of the most suitable equipment to be employed in the anaerobic treatment of a particular substrate strongly depends on the substrate nature and consequently on the limiting steps of the process (*Jenicek* et al., 1993).[8] Therefore, the substrate anaerobic biodegradability and toxicity should be determined in advance and on the basis of the obtained results the selection of the operational parameters should be carried out (*Grady* et al., 1985).[6]

Bioassay techniques for measuring the presence or absence of inhibitory substances are most promising for resolving anaerobic treatment problems, because they are relatively simple and inexpensive, and do not require knowledge of specific inhibitory substances (*Owen* et al., 1979).[12] Also bioassay techniques are essential for determining biodegradability since no chemical procedure is available which distinguishes between biodegradable and non-biodegradable organics (*Gledhill*, 1979).[4] *Owen* et al., 1979,[12] provided the first description of such a test method, drawing on previous gas measurement (*Nottingham* et al., 1969)[11] and incubation bottle (*Miller* et al., 1974)[10] methods. They based their method on measurement of the excess gas volume ($CH_4 + CO_2$) produced after addition of a test chemical to an anaerobic seed incubated in sealed bottles. Subsequently, *Gledhill* improved the method with the goal of defining a simple protocol that should be established by the American Society for Testing Materials (ASTM) as a standard method (*Gledhill*, 1979). Techniques that follow test chemical degradation by the simple measurement of total net gas production, have been proposed as convenient screening tests for assessing anaerobic biodegradation potential under methanogenic conditions (*Shelton* et al., 1984).[14] In 1995 the first International Standard for evaluation of the ultimate anaerobic biodegradability of organic compounds in digested sludge was issued (ISO 11734, 1995).[7] Present tendency of many researchers is to improve and automate the measurement procedure leading to more accurate results and easier measure-

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ment procedure (*Gorris* et al.,[5] 1988; *Massone* et al.,[9] 1996; *Rozzi* et al.,[13] 2000).

The purpose of this work is:

1. to present a new bioassay test for evaluation of anaerobic biodegradability and toxicity of organic compounds using the Micro Oxymax respirometer and TOC analyzer, and

2. to present the use of this method for the proposal of the initial operational parameters of the continuous process treating waste fermentation broth from clavulanic acid production.

The method was also compared to other known methods; to usually used methanogenic activity tests (*Gledhill*,[4] 1979; *Owen* et al.,[12] 1979; *Young* et al.[20] 1993; *Shelton* et al., 1984;[14] *Stephen* et al.,[16] 1984; *Anderson* et al.,[2] 1991; *Zabranska* et al.,[21] 1994; ISO 11734,[7] 1995) and the advantages of this new bioassay test were listed out as stated in the conclusion.

## Materials and methods

The methodology of a new bioassay test using Micro Oxymax respirometer and TOC analyzer to determine anaerobic biodegradability and toxicity, is specified here. Also the important experimental procedures and calculations for testing waste fermentation broth from clavulanic acid production and for the proposal of the waste initial operational parameters of the continuous anaerobic process, are listed bellow.

### Caracteristic and the preparation of the inoculum used

Anaerobic biomass used for the inoculation of the samples was collected from an anaerobic stabilization of waste sludge treating 41 % domestic and 59 % industrial wastewater. Sludge was collected in wide-necked bottles constructed from high density polyethylene and sealed tightly. After transport to the laboratory, pre-digestion of the sludge was carried out to reduce unspecific gas production and to reduce the influence of the blanks. The sludge was digested without the addition of any nutrients or substrates, at 35±2 °C for 7 days. Predigested sludge was than washed, just prior to use, to reduce the $\gamma_{IC}$ content to less than 10 mg $l^{-1}$ in the final test solution, by centrifuging it in sealed tubes at relatively low speed (e.g. 3000 × *g*) for up to 5 minutes. The pellets were than suspended in an oxygen-free test medium, centrifuged and the washing water was discarded. The washing procedure was repeated until the $\gamma_{IC}$ content of the wash water has not been sufficiently lowered (ISO 11734, 1995).[7]

### Caracteristics of the materials tested

Waste fermentation broth had been previously filtered through filters with pore diameter 0,14 mm and then analyzed. Analytical measurements of the tested waste fermentation broth from clavulanic acid production are summarized in Table 1.

Table 1 – *Analytical measurements of the tested waste fermentation broth from clavulanic acid production.*

| Measurement | Quantity | Unit | Value |
|---|---|---|---|
| TOC | $\gamma$ | g $l^{-1}$ | 50.4 |
| pH | $\gamma$ | | 5.12 |
| chloride ($Cl^-$) | $\gamma$ | mg $l^{-1}$ | 68 |
| nitrite-N ($NO_2^-$) | $\gamma$ | mg $l^{-1}$ | 0 |
| nitrate-N ($NO_3^-$) | $\gamma$ | mg $l^{-1}$ | 96 |
| phosphate-P | $\gamma$ | mg $l^{-1}$ | 270 |
| sulfate | $\gamma$ | mg $l^{-1}$ | 50 |
| ammoniacal-N | $\gamma$ | mg $l^{-1}$ | 593 |
| N-tot | $\gamma$ | mg $l^{-1}$ | 3000 |

### Preparation of test and control assays

Test medium containing the constituents as stated in (ISO 11734, 1995),[7] was prepared. Washed predigested sludge was suspended in the requisite volume of test medium to give a concentration of volatile suspended solids in the range of 1 to 3 g $l^{-1}$. All the above operations were carried out in a dry box flushed with nitrogen to assure anaerobic conditions. Homogeneous transfer of the suspension of the test medium and inoculum to the test chambers was ensured by using a peristaltic pump placed in a dry box. The tests were performed in 250 ml vials with a useful volume of 200 ml in triplicates for test compound and blank and 1 vessel each for reference substance and inhibition control (Stergar, 1999).[18]

***Test compound.*** The test compound: waste fermentation broth was added to the mixture of inoculum and test medium to get an initial loading ratio of (0.05; 0.075; 0.30; 0.49; 1.10; 1.7; 2.3 and 4.6) $g_{TOC}\ g_{VSS}^{-1}$.

***Reference substance*:** Phenol was used as a reference substance. Stock solution of phenol was added to the mixture of inoculum and test medium to maintain the concentration of phenol 100 mg $l^{-1}$ TOC.

***Inhibition control.*** Equal mass concentration of test compound and reference substance: 100

mg $l^{-1}$ TOC, were added to a vessel containing test medium. The composition of the test medium is presented in Table 2 and 3.

***Blank vessel.*** Equivalent amounts of anoxic water were added to the mixture of inoculum and test medium.

## Analytical methods

The determination of total suspended solids (TSS) and volatile suspended solids (VSS) was carried out as stated in (APHA, 1998).[1] Respirometric measurements were done on modified Micro Oxymax respirometer for measurements under anaerobic conditions (Columbus Instruments, 1996).[6] Total organic carbon, TOC, inorganic carbon, IC, and total carbon, TC, analyses were obtained using Schimadzu Total Organic Carbon Analyzer model TOC-5000A (Shimadzu Corporation, 1998).[15] Liquid phase was sampled during the assay through septum lid.

## Respirometric measurements

After preparation of the test and control assays, the test vessels were placed in thermostated water bath at 35±2 °C and connected to a Micro Oxymax respirometer to measure the concentration and composition of the gases in headspace volume. Figure 1 shows the Micro Oxymax respirometer in operation under anaerobic conditions. The fraction $O_2$, $CO_2$ and $CH_4$ gas levels of the test chamber environment were measured periodically, and the changes in the levels were used to compute the $CH_4$, $CO_2$ produc-

Table 2 – *Composition of the test medium used in anaerobic bioassay tests.*

| Compound | Mass concentration mg $l^{-1}$ |
|---|---|
| $KH_2PO_4$ | 270 |
| $Na_2HPO_4 \cdot 12H_2O$ | 1120 |
| $NH_4Cl$ | 530 |
| $CaCl_2 \cdot 2H_2O$ | 75 |
| $MgCl_2 \cdot 6H_2O$ | 100 |
| $FeCl_2 \cdot 4H_2O$ | 20 |
| resazurin (oxygen indicator) | 1 |
| $Na_2S \cdot 9H_2O$ | 100 |
| Solution of trace elements | 10 ml $l^{-1}$ |

Table 3 – *Composition of the solution of trace elements used in anaerobic bioassay tests.*

| Compound | Mass concentration mg $l^{-1}$ |
|---|---|
| $MnCl_2 \cdot 4H_2O$ | 50 |
| $H_3BO_3$ | 5 |
| $ZnCl_2$ | 5 |
| $CuCl_2$ | 3 |
| $Na_2MoO_4 \cdot 2H_2O$ | 1 |
| $CoCl_2 \cdot 6H_2O$ | 100 |
| $NiCl_2 \cdot 6H_2O$ | 10 |
| $Na_2SeO_3$ | 5 |

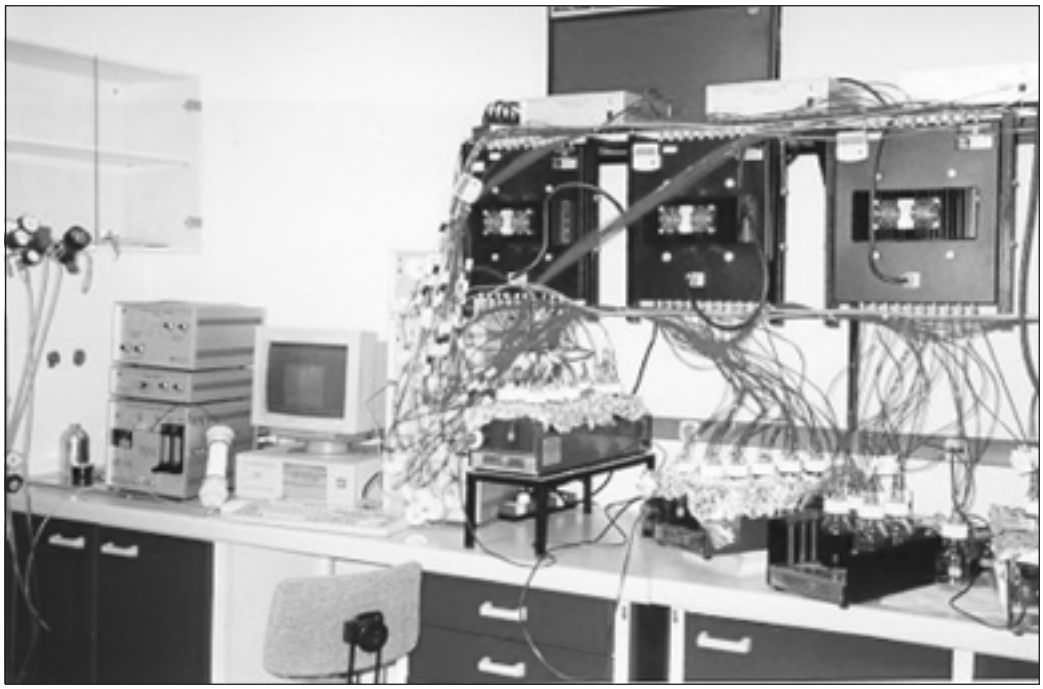


Fig. 1 – *Micro Oxymax respirometer in operation under anaerobic conditions.*

tion and to control the oxygen absence, if anaerobic conditions were given. This system represents a fully automated approach utilizing a microcomputer as a dedicated controller. With the addition of optional Expansion Interfaces, the system was expanded to 40 channels (measuring vessels). The Micro Oxymax respirometer was prior modified to maintain proper measurements under anaerobic conditions by:

(I) connecting the bottled gas with adequate pressure regulators (*nitrogen port*: pressure = 0.054 $10^5$ Pa, gas flow rate = 0.25 – 0.50 l $min^{-1}$; *calibration port:* pressure = 0.72 $10^5$ Pa, gas flow rate = 4-5 l $min^{-1}$) to the system sample pump (Stergar, 1999),[17]

(II) ensuring anaerobic composition of the bottled gas: $\varphi$ = 99.999 % $N_2$, below 2 ppm $O_2$, 5 ppm $H_2O$, 0.5 ppm $H_2$ and 0.5 ppm hydrocarbons,

(III) installing charcoal filters between condensers and dry expansion units to avoid the problems specified in an article (Stergar *et al.,* 1999),[17,18]

(IV) ensuring tight tubing connections and the system without leaking to obtain accurate results and anaerobic conditions (Columbus Instruments, 1996),

(V) ensuring accurate equipment to thermostat sample chambers at 35±2 °C during measurements (*Stergar* et al., 2000),[19]

(VI) changing software settings for operation under anaerobic conditions (Stergar, 1999).[17,18]

### Biodegradation studies

Initial concentrations of waste fermentation broth were obtained as $\gamma_{TOC}$ (mg $l^{-1}$) measurement. The theoretical amount of carbon in produced biogas should be equal to the initial concentration of carbon in the liquid phase at 100 % mineralization. The produced mass or volume of $CO_2$ and $CH_4$ was measured by the Micro Oxymax respirometer and the obtained measurements were calculated to the mass of carbon in produced biogas. The extent of biodegradation ($D_t$) was calculated using equation 1. $D_t$ (%) is the total biodegradation calculated from respirometric and TOC measurements of $CO_2$ and $CH_4$. $\gamma_{TOCinitial}$ and $\gamma_{TOCblank}$ (mg $l^{-1}$) are the initial concentrations of organic carbon in liquid phase of test chamber and blank chamber, respectively. $\gamma_t$ (mg $l^{-1}$) is the total mass of carbon in liquid and gas phases per volume of liquid phase, calculated from respirometric and $\gamma_{TOC}$ measurements. $\gamma_h$ (mg $l^{-1}$) is the mass of produced carbon in biogas per volume of liquid phase, and $\gamma_l$ (mg $l^{-1}$) is the concentration of dissolved inorganic carbon in the liquid phase. $\gamma_{ICsample}$ and $\gamma_{ICblank}$ (mg $l^{-1}$) are the values of inorganic carbon of the test chamber and blank chamber, respectively.

$$D_t = \frac{\gamma_t \cdot 100}{\gamma_{TOC_i} - \gamma_{TOC_b}} \quad (1)$$

$$\gamma_t = \gamma_h + \gamma_m \quad (2)$$

$$\gamma_t = \gamma_{ICs} - \gamma_{ICb} \quad (3)$$

$$\gamma_h = \frac{\gamma_{CH_4} \cdot M_C}{M_{CH_4}} + \frac{\gamma_p \cdot M_C}{M_{CO_2}} \quad (4)$$

$$\gamma_{CH4} = \gamma_{CH4resp.} - \gamma_{CH4blank} \quad (5)$$

$$\gamma_p = \gamma_{CO2resp.} - \gamma_{CO2blank} \quad (6)$$

$\gamma_{CH4}$ (mg $l^{-1}$) is the cumulative value of $CH_4$ production per volume of liquid phase, $\gamma_{CH4resp.}$ and $\gamma_{CH4blank}$ (mg $l^{-1}$) are cumulative values of produced $CH_4$ per volume of liqud phase measured in test chamber and blank chamber, respectively. $\gamma_p$ (mg $l^{-1}$) is the cumulative value of $CO_2$ produced in headspace gas per volume of liquid phase and measured by Micro Oxymax respirometer, $\gamma_{CO2resp.}$ and $\gamma_{CO2blank}$ (mg $l^{-1}$) are the cumulative values of produced $CO_2$ measured in headspace gas of the test and blank chamber, respectively and calculated per volume of liquid phase. $M_{CO2}$, $M_{CH4}$ and $M_C$ are the molar masses of $CO_2$, $CH_4$ and carbon, respectively. Biodegradation curves were plotted as % of degradation versus time.

The volume or mol fraction of methane gas in produced biogas was calculated by equation 7. In the calculation the following should be considered: measurements were done at constant pressure and temperature and only methane and $CO_2$ represent the composition of produced biogas.

$$\varphi_{CH_4} = \frac{2.75 \cdot \zeta}{2.75 \cdot \zeta + 1} \quad (7)$$

$$\zeta_{CH_4/CO_2} = \frac{m_{CH_4}}{m_{CO_2}} \quad (8)$$

$$V = \frac{n \cdot R \cdot T}{p} \quad (9)$$

Where $V$ is volume of gas ($m^3$), $R$ is constant for gas (8.314 J $mol^{-1}$ $K^{-1}$), $p$ is pressure of the gas (Pa), $T$ is temperature (K), $n$ is amount of the gas (mol), $\varphi_{CH4}$ is volume fraction of methane gas (%) and $\zeta_{CH4/CO2}$ is mass ratio of methane and $CO_2$.

## Results and discussion

Table 4 summarizes the operational parameters of the tests and the data measured and calculated. The process was optimized for two degradation efficiencies 70 % and 85 %. Degradation efficiency

Table 4. – *Test parameters.*

| Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| TOC initial, mg $l^{-1}$ | 100 | 150 | 600 | 990 | 2200 | 3400 | 4600 | 9200 |
| initial loading, $\gamma_{TOC}$ $g_{VSS}^{-1}$ | 0.05 | 0.075 | 0.30 | 0.49 | 1.10 | 1.70 | 2.30 | 4.60 |
| cumulative biogas production mg $l^{-1}$ C | 92 | 141 | 562 | 1002 | 1980 | 3067 | 3312 | 2843 |
| TOC biodegradability: $D_t$, % | 92.0 | 94.0 | 93.6 | 93.5 | 90.0 | 90.2 | 72.0 | 30.9 |
| $\varphi_{CH4}$ in biogas, % | 54 | 56 | 65 | 72 | 71 | 70 | 67 | 50 |
| time of 70% efficiency, d | 10 | 9 | 10 | 13.5 | 23 | 36 | 57 | |
| time of 85% efficiency, d | 20 | 16.5 | 18.5 | 21 | 29 | 41 | 70 | |
| loading rate Bx at 70% efficiency, $g_{TOC}$ $g_{VSS}^{-1}$ $d^{-1}$ | 0.005 | 0.008 | 0.030 | 0.036 | 0.048 | 0.047 | 0.040 | |
| loading rate Bx at 85% efficiency, $g_{TOC}$ $g_{VSS}^{-1}$ $d^{-1}$ | 0.003 | 0.0045 | 0.016 | 0.023 | 0.038 | 0.041 | 0.033 | |

70 % was considered as the minimum requirement of the operational parameters for the running process and 85 % as the optimum of the biodegradation process. The retention times needed for a 70 % and 85 % degradation efficiency were evaluated from the curves of the extent of biodegradation $D_t$ (%) as a time when the biogas production ($CO_2$ and $CH_4$) reached the 70 and 85 % of the total production, respectively. These data were used for the starting up biomass loading rate $B_x$ ($g_{TOC}$ $g_{VSS}^{-1}$ $d^{-1}$) of a continuous operation assuming 70 % and 85 % efficiency of $\gamma_{TOC}$ removal desired.

From Figure 2 and 3 it is seen that the increased initial concentration of waste fermentation broth caused the increase in cumulative production of $CO_2$ and $CH_4$ and higher rates of biogas production.

The relation of the total biogas produced (expressed as mg carbon per liter) per gram of volatile suspended solids ($\gamma_{VSS}$) to the initial substrat loading is not linear over the whole range of loadings (Figure 4), hence the degradation was limited by the amount of substrates at low initial loading ratios below 0.5 $g_{TOC}$ $g_{VSS}^{-1}$. This conclusion resulted also from Figures 2 and 3. From the Figure 4 and 5 it is obvious that the substrate did cause an inhibition at higher initial loading ratios (2.3 $g_{TOC}$ $g_{VSS}^{-1}$) and a very distinctive fall of cumulative biogas production at initial loading ratio above 4.6 $g_{TOC}$ $g_{VSS}^{-1}$.

Retention times of 70 % and 85 % degradation as a function of the initial loading ratio are shown in Figure 6. Initial biomass loading ratios below 0.3 $g_{TOC}$ $g_{VSS}^{-1}$, resulted to a decrease of the retention times with increased loadings. Thus the degradation was at low loading rates limited by the amount of substrate.

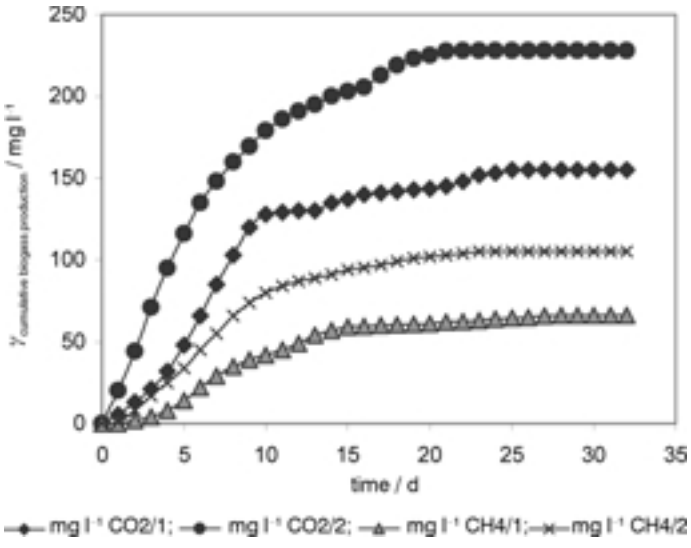



Fig. 2 – *Cumulative biogas production of $CO_2$ and $CH_4$ of two samples of waste fermentation broth with different initial concentration of total organic carbon: sample 1: $\gamma_{TOCinitial}$ = 100 mg $l^{-1}$, sample 2: $\gamma_{TOCinitial}$ = 150 mg $l^{-1}$. Dissolved $CO_2$ in liquid phase was measured by $\gamma_{TOC}$ analyser as $\gamma_{IC}$ value and added to cumulative production of gas $CO_2$ measured by Micro Oxymax respirometer.*

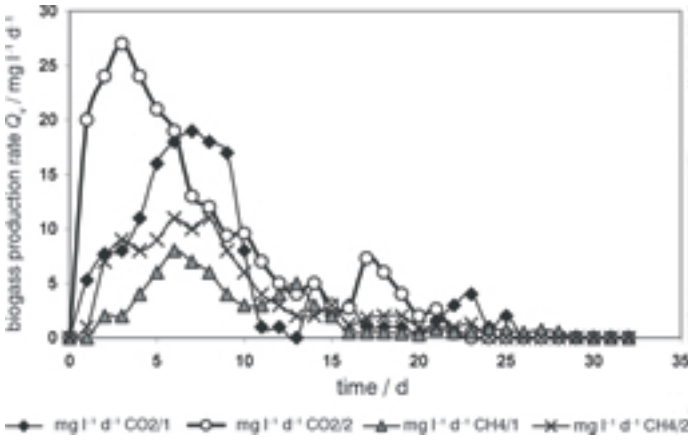
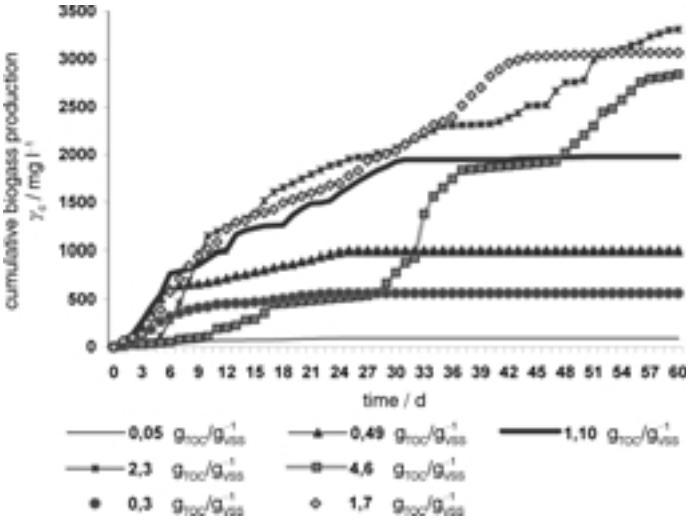
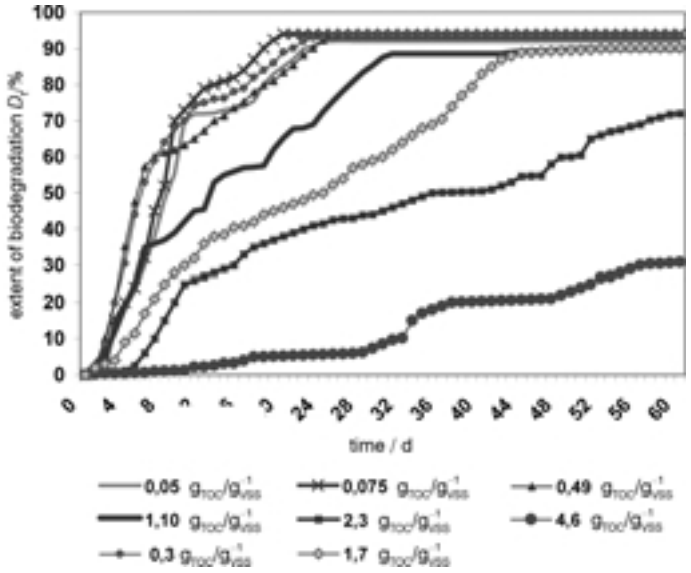


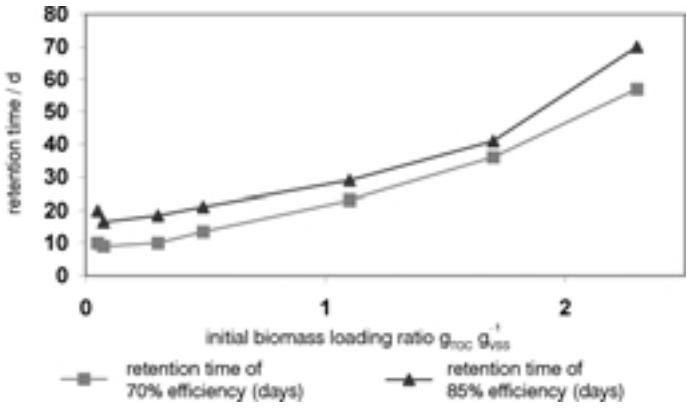

Fig. 3 – *Methane and $CO_2$ production rate of waste fermentation broth with different initial concentration of total organic carbon: sample 1: $\gamma_{TOCinitial}$ = 100 mg $l^{-1}$, sample 2: $\gamma_{TOCinitial}$ = 150 mg $l^{-1}$.*




F i g . 4 – *Cumulative $CO_2$ and $CH_4$ production expressed as mg of total carbon per liter of the suspension of inoculum and test medium (endogenous production substracted), measured by Micro-Oxymax respirometer.*



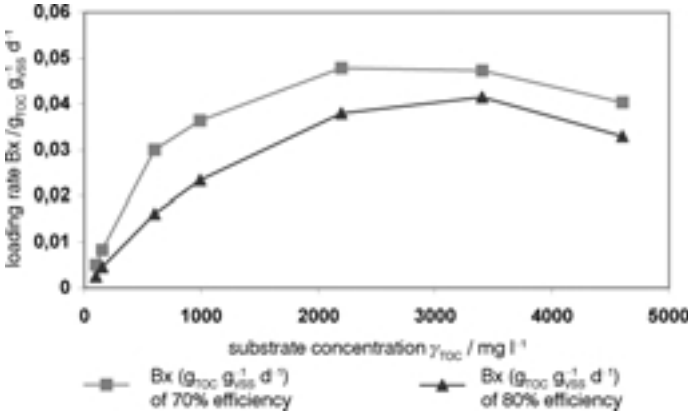

F i g . 5 – *The extent of biodegradation $D_t$ (%) of waste fermentation broth at different initial biomass loading ratios (0.05-4.6 $g_{TOC}\ g_{VSS}^{-1}$). $D_t$ was calculated from data presented in Figure 4. using equation 1.*




F i g . 6 – *The retention times of 70 % and 85 % degradation efficiency as a function of the initial biomass loading.*

The functions on Figure 6, for 70 % and 85 % efficiency, are close to linear from the initial loading ratio 0.3 $g_{TOC}\ g_{VSS}^{-1}$ up to a loading of 1.7 $g_{TOC}\ g_{VSS}^{-1}$, but these retention times were prolonged at higher values of loading. This means that the degradation rate is slower at a high concentration of the substrate.

The same conclusion results from Figure 7, where the dependence of loading rates, $B_x$ ($g_{TOC}\ g_{VSS}^{-1}\ d^{-1}$) upon substrate concentration is presented. The optimum biomass loading rate for the start-up operation is 0.042 $g_{TOC}\ g_{VSS}^{-1}\ d^{-1}$ at 85 % efficiency and 0.048 $g_{TOC}\ g_{VSS}^{-1}\ d^{-1}$ at 70 % efficiency. The treatment of the substrate with a concentration higher than 3400 mg $l^{-1}$ TOC needs a longer retention time, and it is therefore necessary to decrease the biomass loading rate for the same TOC removal efficiency.




F i g . 7 – *The dependence of loading rates upon substrate concentrations.*

This is valid for the start-up procedure, but after the adaptation of biomass the optimum substrate concentration may be increased. The batch tests give some links to the start up procedure of a continuous fed reactor, but it is not possible to make an exact extrapolation from batch to a continuous fed systems.

## Conclusions

A recently developed method for evaluating anaerobic biodegradability using Micro Oxymax respirometer and TOC analyzer was in this case successfully used for the determination of anaerobic biodegradability of a pharmaceutical waste - waste fermentation broth from clavulanic acid production and mass balance for carbon was monitored during anaerobic biodegradation process was monitored. The process was optimized for two degradation efficiencies 70 % and 85 %. Degradation efficiency of 70 % was considered as the minimum requier-

ment of the operational parameters for the running process, and 85 % as the optimum of the biodegradation process.

Retention times, needed for 70 % and 85 % degradation efficiency, were evaluated from the tests with different initial substrate concentrations, and were used for the assessment of the starting-up substrate loading rate of the continuous reactor operation - 0.042 $g_{TOC}\ g_{VSS}^{-1}\ d^{-1}$ for 85 % efficiency and 0.048 $g_{TOC}\ g_{VSS}^{-1}\ d^{-1}$ for 70 % efficiency.

The optimum substrate concentration was estimated as a maximum value from the plot of loading rates $B_x$ against the substrate concentration and it was 2.2 g $l^{-1}$ TOC for 70 % efficiency and 3.4 g $l^{-1}$ TOC for 85 % efficiency.

The volume fraction of the methane in produced biogas was determined for each initial loading ratio. Methane gas presented around 70 % of total biogas volume at initial substrate loading ratios between 0.49 and 2.3 $g_{TOC}\ g_{VSS}^{-1}$.

The results of the tests proved the usefulness of this method for the determination of anaerobic biodegradability and the yield of methane in produced biogas, and for the proposal of operational parameters of the continuous process.

The use of this new technique leads to the following advantages over the other known procedures (*Gledhill*[4], 1979; *Owen* et al.,[12] 1979; *Young* et al.,[20] 1993; *Shelton* et al.,[14] 1984; *Stephen* et al., 1984;[16] *Anderson* et al.,[2] 1991; *Zabranska* et al.,[21] 1994; ISO 11734,[7] 1995):

– The method ensures automatic, simultaneous measurement of many parameters during the assay.

– The method provides more accurate results than the method described in (ISO 11734, 1995).[7] The comparison of the results was presented and statistically evaluated in (*Stergar*, 2000).[19]

– Continuous, automatic measurements of the $CO_2$ and $CH_4$ separately give more insight into the kinetics of anaerobic biodegradation (*Stergar*, 2000).[19]

– Septum lid option provides simultaneous measurements of liquid and gas phases resulting in determination of dissolved $CO_2$ during the assay.

– TOC analyses of the initial loading, instead of COD as used in (*Zabranska* et al., 1994),[21] and following the biodegradation by the mass balance of carbon, give more details about each step of the biodegradation process.

## *ACKNOWLEDGEMENTS*

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## List of symbols

*ASTM* – American Society for Testing Materials

$B_x$ – biomass loading rate, $g_{TOC}\ g_{VSS}^{-1}\ d^{-1}$

$\gamma_{CH4}$ – cumulative value of $CH_4$ production per volume of liquid phase, mg $l^{-1}$

$\gamma_{CH4blank}$ – cumulative value of produced $CH_4$ per volume of liqud phase measured in blank chamber, mg $l^{-1}$

$\gamma_{CH4resp.}$ – cumulative value of produced $CH_4$ per volume of liqud phase measured in test chamber, mg $l^{-1}$

$\gamma_{CO2blank}$ – cumulative value of produced $CO_2$ measured in headspace gas of the blank chamber, mg $l^{-1}$

$\gamma_{CO2resp.}$ – cumulative value of produced $CO_2$ measured in headspace gas of the test chamber, mg $l^{-1}$

$\gamma_p$ – cumulative value of $CO_2$ produced in headspace gas per volume of liquid phase, mg $l^{-1}$

$D_t$ – extent of biodegradation, %

$\zeta_{TOC/VSS}$ – substrate loading ratio, gram total organic carbon per gram volatile suspended solids, $g_{TOC}\ g_{VSS}^{-1}$

$m_{TOC}\ m_{VSS}^{-1}\ t^{-1}$ – gram total organic carbon per gram volatile suspended solids, per day, g $g^{-1}$ $d^{-1}$

$\gamma_{ICb}$ – concentration inorganic carbon of the blank chamber, mg $l^{-1}$

$\gamma_{ICs}$ – concentration inorganic carbon of the test chamber, mg $l^{-1}$

$M_C$ – molar mass of carbon, g $mol^{-1}$

$M_{CH4}$ – molar mass of $CH_4$, g $mol^{-1}$

$m_{CH4}$ – mass of $CH_4$, g

$M_{CO2}$ – molar mass of $CO_2$, g $mol^{-1}$

$m_{CO2}$ – mass of $CO_2$, g

$\gamma_C$ – mass concentration of carbon, mg $l^{-1}$

$\gamma_h$ – mass concentration of produced carbon in biogas per volume of liquid phase, mg $l^{-1}$

$\gamma_{ICl}$ – concentration of dissolved inorganic carbon in the liquid phase, mg $l^{-1}$

$\zeta_{CH4/CO2}$ – mass ratio of methane and $CO_2$

$\gamma_{e,t}$ – total mass of carbon in liquid and gas phases per volume of liquid phase, mg $l^{-1}$

$n$ – amount of the gas, mol

$\gamma_{Nt}$ – total mass concentration of nitrogen, mg $l^{-1}$

$p$ – pressure of the gas, Pa

$R$ – gas constant (8,314 J $mol^{-1}$ $K^{-1}$

$T$ – temperature, K

$\gamma_{TOC}$ – mass concentration of total organic carbon, mg $l^{-1}$

$\gamma_{TOCb}$ – initial concentrations of organic carbon in liquid phase of blank chamber, mg $l^{-1}$

$\gamma_{TOCi}$ – initial concentrations of organic carbon in liquid phase of test chamber, mg $l^{-1}$

$V$ – volume of gas, $m^3$

$\gamma_{CH4}$ – volume percent of methane gas (%)

$\gamma_{VSS}$ – volatile suspended solids, g $l^{-1}$

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