

ENZYME ACTIVITY ANALYSES OF ANAEROBIC FERMENTED SEWAGE SLUDGES

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Abstract. Biogas may be produced from communal sewage sludges by anaerobic decomposition. To follow up the decomposition processes enzyme activity tests have been applied. It is advantageous to use these activity tests if there are frequent substrate-, or specific organic matter load changes in the digesting towers. For describing the total activity of the cells, dehydrogenase-, while for following up the frequent substrate changes protease-, lipase- and cellulase enzyme activity analyses have been carried out. No internationally accepted standards are available for testing the enzyme activity of sewage sludges. Thus, in our experimental work we modified the enzyme activity analyses described earlier in the special literature for the tests of other than sewage sludge samples. Our enzyme activity tests can be completed quickly and at a relative low cost, thus it is possible to use them in generally equipped wastewater treatment laboratories also.

Keywords: *sludge, dehydrogenase, lipase, protease, cellulase*

Introduction

In a communal wastewater treatment plant large volumes of sewage sludge occur from which it is possible to produce biogas by anaerobic fermentation. The energetical use of the produced biogas takes a part in the increasing power independence of the plant as burning the produced biogas the total electric power demand of the plant can be met. Treated or untreated sewage sludge can be made extensive use like agricultural soil improving material too (Yudhistrá Kumar and Vikram Reddy, 2010). Biogas is a very important renewable energy source in the world. For example in India the government sponsors the building of further 3 million family scale biogas reactors by 2012 (Raghuvanshi et al., 2008). For this reason an increase both in biogas volume and in heating power is an important goal, and may be answered if the anaerobic fermentation process is understood more thoroughly. In the course of our research the enzyme activities of sludge samples originating from anaerobic sewage sludge fermentation plants were studied under different operating conditions – which were described in our former article (Kardos et al., 2009). In the present article enzyme activity

measurements used for following up the anaerobic decomposition are described in more details.

With the enzyme activity measurements the initial hydrolysis processes of the anaerobic decomposition can be described – being also the rate determining step of the complete process (Thiel et al., 1968).

Material and methods

The sludge samples originated from the South-Pest Wastewater Treatment Plant (Budapest, Hungary) where communal wastewater is treated. The Plant has a capacity of 80000 m³/day. One thermophilic, three mesophilic anaerobic full scale fermenters and three pilot plant scale fermenters can be found in the Plant. The thermophilic plant tower is of 2000 m³ while each mesophilic tower has a capacity of 2600 m³. The average operating temperature of the thermophilic tower is 55°C and it is 35°C in the case of the mesophilic towers. The useful capacity of the pilot plant scale fermenters is 1.5, 2.0 and 2.5 m³ respectively, and their temperature can be varied. For our experiments the anaerobic sludge was taken from all these fermenters, however here only some measured data are shown – as examples – related to sludge samples derived from the pilot plant scale fermenters.

Our enzyme activity analyses covered dehydrogenase enzyme activity typical of the total activity of the cells, and three substrate specific enzyme activities; i.e. protease-, lipase- and cellulase enzyme activities. The analysis of the substrate specific enzyme activities was reasoned by the frequent changes in the protein- and fat content of the sludge. The cellulase enzyme activity was analysed only in those cases when the sewage sludge and the horticultural wastes had been fermented together (studying co-substrate effect).

There are no internationally accepted standards for the enzyme activity tests of the anaerobic sewage sludge samples, thus our used formulas were based on earlier prescriptions. Each enzyme activity measurement was thus optimised in our adopted recipes related to the applied temperature, incubation period and also the sludge mass (latter expressed as dry material /reagent unit ratio). The blank samples composition and role were also investigated, and the proper one was selected for each enzyme activity test type.

The basis for the measurement of the *dehydrogenase* enzyme activity typical of the total activity of the cells was provided by the Hungarian Standard MSZ-08-1721/3:1986. As this standard described the determination of the actual dehydrogenase enzyme activity of soil samples, we adapted the method of this measurement to anaerobic sludge samples. In the course of our work the testing results of Skujins (1976), García et al. (1993) and Griebel et al. (1997) were also used. The principle of the measurement is – as a result of the process catalyzed by the enzyme – that 2,3,5-triphenyltetrazolium chloride (TTC) transforms to a red colour triphenyl-phormazane (TP), the quantity of which could be spectrophotometrically measured. Knowing the measured sludge quantity, the produced triphenyl-phormazane concentration is converted to specific units of mg TP/g organic matter/hour.

To follow up the enzyme activity, 1 cm³ TTC-solution of 1 m/m% was added to the sludge samples in addition to the saturated NaHCO₃ puffer solution. Sludge samples of 0.5, 1.0, 2.0 and 4.0 cm³ were used in our series of experiments. Each case the blank sample was further treated in the same way but of course no substrate had been added

yet. Also the effect of a change in the incubation temperature was analysed. The original recipe suggested a temperature of 37°C. For studying the dependence on temperature, four temperatures were selected; i.e. room temperature (20°C), 37°C (temperature of the mesophilic sludge treatment), 45°C and 55°C (temperature of the thermophilic sludge treatment).

We analysed the incubation period too. The incubation period was varied between half an hour to four hours, after which the enzyme activity of each sample was stopped by absolute ethanol. Immediately before ethanol addition, 1 cm³ substrate was added to the blank samples. After 10 minutes of sedimentation, the samples were filtered and the red solutions were photometered at 485 nm against ethanol.

The principle of the *protease* enzyme activity measurement is the follow up of the protein (casein) transition to amino acid (tyrosine). The protease activity measurement of sewage sludges was described by Thiel and Hattingh (1967). In our series of experiments each sample contained 1/3 volume part of sludge sample, 1/3 volume part substrate (0.25 m/m% casein-solution), and 1/3 volume part distilled water. After incubation at room temperature for 1 hour (according to the original recipe) the reaction was stopped by trichloroacetic acid. After alkalization following filtering – due to the separating iron precipitate – it was filtered again and after adding Folin-reagent to the sample the appearing blue colour was measured at 660 nm in comparison to the blank sample. The anaerobic sludge treated in the above ratios without incubation served as a blank sample following the immediate trichloroacetic acid treatment. The measured data are expressed in mg tyrosine/g organic matter/hour units.

Also in the case of the protease enzyme activity tests, the volume of the sludge sample was changed (0.5, 1.0, 2.0 and 4.0 cm³). In another test series the incubation temperature was altered; in this case also the applied temperatures were the followings: room temperature (20°C on an average); 37°C; 45°C, and 55°C. The incubation period was selected between half hour and four hour. In addition the appropriate concentration of the trichloroacetic acid and the rate of the alkalization following the precipitation with trichloroacetic acid were also analysed. We adjusted the optimum pH of the samples. The blue colour appears immediately after the addition of the Folin-reagent and can be measured at 660 nm. Also the time dependence of the blue colour stability was tested when the intensity variation of the blue colour was being studied for 40 minutes.

For measuring *lipase* enzyme activity, the work of Vorderwülbecke et al. (1992) and Li and Chróst (2006) were used as a basis. For the measurement, the emulsion of two reagent (containing 4-nitrophenyl palmitate -NPP- as substrate) solutions had to be prepared that was added to a predetermined portion of the supernatant layer of the centrifuged sludge. After one hour incubation at 45°C (according to the original recipe), the absorption of the sample was measured at 410 nm in comparison with the blank sample. In the course of the enzyme processes, the 4-nitrophenyl palmitate – being present as a substrate – was transforming to 4-nitrophenol (NP) that could be measured spectrophotometrically. The activity was expressed in 4-nitrophenol mass produced hourly by 1 g organic sludge. Improving the recipe in this case also the incubation temperatures and the change of incubation period as described above were studied.

In measuring the *cellulase* enzyme activity, the work by Lee et al. (2002) and Rivard et al. (1994) served as a starting point. In the course of the measurement, the cellulose decomposition of the fermented sludge was examined at 37°C for an incubation period of 24 hours according to the original recipe. Each case an easily decomposing cellulose

by-product (carboxymethyl cellulose, CMC) and ground grass dried to a mass steadiness – that can be decomposed with more difficulty – were added to a portion of the sludge sample. After incubation the suspension was centrifuged. In the centrifuged product the glucose - liberated from the cellulose products - was determined with the addition of a dinitro-salicylic acid (DNS) reagent. The sample got brown to an extent which depended on its reducing sugar content and was photometered at 550 nm. Glucose originating from the decomposition of the sludge was corrected with a blank sample containing no substrate. The evaluation was made with the aid of glucose calibration. The activity was stated in glucose quantity liberated daily by a unit of organic sludge.

For improving the recipe, in this case also the incubation temperatures and the change of the incubating period were studied.

Results

Among the results presented in the followings, data of minimum 25 samples referring to each test series are described. The examined sludge had an average pH = 7.1 $\pm$ 0.5, average dry material content 56.8 $\pm$ 9.6 g/kg, average organic material content 26.7 $\pm$ 7.6 g/kg.

During the analysis of the *dehydrogenase* enzyme activity rising the volume of the sludge samples – that is sludge mass ratio related to the applied reagent quantity – a nonlinear increase in the measured absorbances was observed. We stated that in the case of 1 cm³ substrate solution (1 mm% TTC) the reliable sludge volume was 1cm³ testing sludge samples having the earlier mentioned organic mass concentration. In line with raising the incubating temperature an increasing enzyme activity was indicated by the higher absorbance values. From our results it was concluded that this enzyme tests can be made at any temperature between 30 and 55°C. In mesophilic anaerobic fermentation the incubation at about 37°C, while in thermophilic fermentation incubation at about 55°C is suggested, as these temperatures are near to the optimal life conditions of the mesophilic and thermophilic bacteria. The optimal incubation time determined at each temperature turned to be 1 hour.

On the basis of our *protease* activity measurement, in the case of the used substrate concentration, the 2 cm³ sludge sample volume having the given mass concentration the most suitable. On the basis of our experiences – independently of the mesophilic or thermophilic origin of the sludge – considering both the activity and the reproducibility - the most advantageous temperature turned to be 37°C. The 3 cm³ 10 m/m% trichloroacetic acid concentration included in the original recipe was not adequate, thus a concentration of 15 m/m% was used. Due to application of both acid and Folin-reagent (diluted to 1:1), the samples had to be alkalized, thus 3 cm³ 2 mol/dm³ NaOH solution was used before adding the Folin-reagent. The ratio of Folin-reagent and sample was in volume: 0.1: 1. On the basis of the time dependence analysis for the occurring blue colour it can be stated that the blue colour had been stabilized after a period of approximately 15 minutes.

As a result of the *lipase* enzyme activity tests it can be stated that - due to the activity and the reproducibility data - the suitable incubation temperature is 45°C, and it does not depend on the preliminary fermentation temperature. The optimum incubation time was between 60 and 80 minutes.

Our *cellulase* analyses proved that the suitable incubation temperature is 37°C. We suggest an incubation period of 24 hour which can be appropriately applied also by an average laboratory of a wastewater treatment plant. The incubation period of 24 hours is reasoned by the fact that during this period the hydrolysis of cellulase molecules – which can be decomposed with difficulty - will be suitably completed.

Here we show some results of vegetable waste and sludge cofermentation (cosubstrate effect) from our pilot plant scale experiments. It can be seen, that during the experiment the cellulase- and protease enzyme activities showed a close correlation with the alteration of the daily gas volume (*Figure 1*). The changes in substrate load were properly indicated by our enzyme activity measurements (organic matter load varied: $5.24 \pm 1.4 \text{ kg/m}^3 \cdot \text{d}$). The supply of the grass waste was preceded by a control period of 36 days during which only sludge was added. The control period was followed by a grass supplying period of 76 days meanwhile each working day 1.5 kg dried ground grass together with 200 dm^3 sludge was added. On the basis of our tests it can be stated that the simultaneous sludge and grass feeding presented suitable nutriment for the anaerobic microbe population.

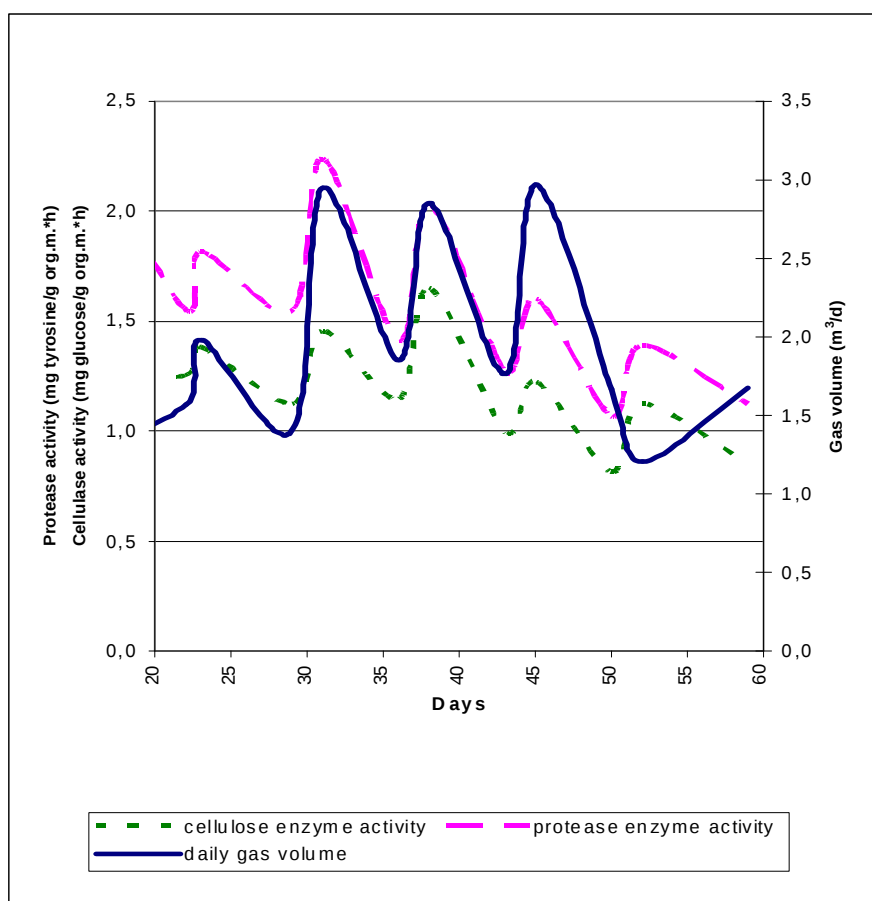


Figure 1. Variation of cellulase and protease enzyme activities in a selected experimental period in pilot plant scale reactor (org.m. = organic material)

Our observations supported that the decomposability of a substrate can be described by the hydrolytic enzyme activity as the speed of the substrate decomposition is the function of enzyme activity, and this is an important condition for biogas

transformation. More results of full scale and pilot plant fermentation are published elsewhere (Kardos et al, 2009).

Summary

As a summary our experiences on the recipe modifications are described in *Table 1*.

Table 1. Summary of the enzyme activity tests (tested sludge: $pH = 7.1 \pm 0.5$, average dry material content 56.8 ± 9.6 g/kg, average organic material content 26.7 ± 7.6 g/kg)

Enzyme	Substrate	Product of metabolism	Sludge volume (cm ³)	Incubation temperature	Others
Dehydrogenase	2,3,5-triphenyl-tetrazolium chloride (TTC)	triphenyl-phormazane (TP)	1	In case of a mesophilic sludge: 37°C, in case of a thermophilic sludge: 55°C	Incubation for 1 hour
Protease	casein	tyrosine	2	37°C, independently of the preliminary sludge fermentation temperature	Treatment with 15 m/m% trichloroacetic acid and 2 mol/dm ³ NaOH Incubation for 1 hour
Lipase	4-nitrophenyl palmitate (NPP)	4-nitrophenol (NP)	1 (liquid extract of sludge)	45°C	Incubation between 60 and 80 minutes
Cellulase	Carboxymethyl cellulose (CMC) or grass	glucose	2 (liquid extract of sludge)	37°C	Incubation for 24 hours

In case of the substrate specific enzyme activity analyses, the stated incubation temperatures have not shown any relationship with the preliminary fermentation temperature of the sludge; it is explained by the fact that in these cases the fat-, protein- and cellulose contents of the sludge are more determinative.

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