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Activity, life time and effect of hydrolytic enzymes for enhanced biogas production from sludge anaerobic digestion

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ABSTRACT

As an alternative to energy intensive physical methods, enzymatic treatment of sludge produced at wastewater treatment plants for increased hydrolysis and biogas production was investigated. Several hydrolytic enzymes were assessed with a focus on how enzyme activity and life time was influenced by sludge environments. It could be concluded that the activity life time of added enzymes was limited (<24 h) in both waste activated sludge and anaerobic digester sludge environments and that this was, for the majority of enzymes, due to endogenous protease activity. In biogas *in situ* experiments, subtilisin at a 1% mixture on basis of volatile solids, was the only enzyme providing a significantly increased biomethane production of 37%. However, even at this high concentration, subtilisin could not hydrolyze all available substrate within the life time of the enzyme. Thus, for large scale implementation, enzymes better suited to the sludge environments are needed.

1. Introduction

The total production of sludge at wastewater treatment plants (WWTP) treating urban wastewater in Europe alone is huge, and by combining the latest reported numbers for each country in EU (2012–13) it can be concluded that approximately 13 million metric tons of sludge dry matter is produced annually (Eurostat, 2016). The organic part of the produced sludge could be used for energy generation, but instead means for sludge disposal ranges from high value energy production to detrimental landfilling or even dumping at sea (Eurostat, 2016). A simple calculation, assuming a reasonable 65% organic content (volatile solids, VS), a biochemical methane potential of 200 Nm^3/ton VS and a degree of degradation of 50% gives that 845 million Nm^3 of biomethane could be produced annually from the sludge generated in Europe. This corresponds to 721 000 tonne of oil equivalents (TOE). However, during the same period (2013) only 125 000 TOE of biomethane was produced from urban, and including industrial, wastewater treatment plants (EurObserv'ER, 2014) and the energy production potential of sludge is thus greatly underutilized. This is unfortunate since wastewater sludge production continuously provides an organic material for anaerobic digestion that does not compete with other uses, as is otherwise the case with e.g. energy crops. The incentive for investment in anaerobic digestion and biogas production at WWTP is however devalued by the low degree of degradation (yield) of WWTP sludge, often around or below 50%.

The sludge produced at WWTP is mainly the sedimented primary sludge and the waste activated sludge (WAS) of biological treatment. The produced sludge can be degraded and reduced through anaerobic digestion which can be summarized to four main stages; hydrolysis, acidogenesis, acetogenesis and finally methanogenesis. Extracellular enzymes, secreted by the microorganisms present in the anaerobic digester are essential for hydrolysis of the particulate bioorganic molecules into monosaccharides, amino acids and fatty acids (Christy et al., 2014; Vavilin et al., 2008). The substrate is thereby made accessible as nutrition for the microorganisms in the anaerobic digester and is metabolized in

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several steps to the end products methane and carbon dioxide (Pavlostathis and Giraldogomez, 1991; Weiland, 2010).

Earlier results have concluded that it is the WAS that is the sludge fraction that is the most recalcitrant to hydrolysis (Gossett and Belser, 1982) and for which the hydrolysis is considered to be the rate limiting step, resulting in the low degree of degradation (Eastman and Ferguson, 1981). The low degradation is also partly due to the inherently short hydraulic retention time (HRT) of the material in anaerobic digesters at WWTPs. Therefore, to increase the degree of degradation within the available retention time, the hydrolysis rate of the substrate has to be improved (Eastman and Ferguson, 1981). To increase the solubilization of the substrate and thereby increase the biogas production, with a concomitant decrease of the amount of sludge for disposal, many chemical and physical, including thermal, pretreatment methods have been evaluated. However, since the dry content of untreated sludge produced at WWTP is only between 0.5% and 3%, much of the required energy input, or addition of chemicals, are therefore unproductive since it is to the largest part water that is being treated. This connection between sludge concentration and pretreatment energy self-sufficiency of various methods has recently been verified (Cano et al., 2015).

To avoid the unproductive treatment of large volumes of water, an attractive alternative method could instead be to use enzymes as they are both biological catalysts and active at mild aqueous conditions, and further active specifically against the substrate itself (Parawira, 2012). Therefore, enzymes can potentially be used without any additional energy input or changes to the conditions of the substrate. However, that potential is only valid under the prerequisite that the enzymes added have a high activity and a long enough life time at the conditions of use. The use of enzymes to increase the accessibility of organic substances has been evaluated for a number of substrates, of which some have been sludges from WWTPs (Davidsson et al., 2007; Diak et al., 2012; Luo et al., 2011). However, previous studies of enzymatic sludge pretreatment have shown diverse results, ranging from no improvements even in soluble chemical oxygen demand (SCOD) (Diak et al., 2012) to increased biogas production (Davidsson et al., 2007; Luo et al., 2011). Other studies have shown improvements on the degradation of digester material upon addition of enzymes, however, at enzyme concentrations that are not economically feasible (Binner et al., 2011).

In order to reach an effect, an important objective is to select enzymes which have a natural substrate in the sludge. According to earlier studies, the WAS consists of the microbial biomass and extracellular polymeric substances (EPS) comprising polysaccharides, proteins, humic substances, uronic acids and deoxyribonucleic acids (Dey et al., 2006). It has further been concluded that the dominant organic content of total WAS is made up of proteins in the range of 40–60% of the COD (Tanaka et al., 1997; Donoso-Bravo et al., 2011) followed by lipids and polysaccharides at approximately 25% and 15%, respectively (Wilson and Novak, 2009). Studies of the effect of various enzymes have been carried out both as pretreatment experiments (Parmar et al., 2001) and as *in situ* treatment (Recktenwald et al., 2008). The studies have resulted in observed improved sludge solubilization by 54.24% upon addition of α-amylase (Yang et al., 2010), a high percentage of reduction of protein upon addition of trypsin (Parmar et al., 2001), and addition of subtilisin (alcalase) increased the SCOD (Nagel et al., 1992). A combination of several different glycosidic enzymes consisting of dextranase and cellulase among others resulted in 60% increased biogas production (Davidsson et al., 2007) and a combination of several glycosidic enzymes resulted in decreased sludge volumes and increased methane production (Recktenwald et al., 2008).

Due to the varied results from earlier studies, the aim of the present study was to complement earlier studies by not solely looking at how the enzymes influence sludge solubilization and biogas production, but more importantly also the reverse, i.e. how is activity and life time of added enzymes influenced by the sludge environments? Two scenarios were analyzed, one in which the enzymes should be used for pretreatment of the recalcitrant WAS, and another in which the enzymes should be used for *in situ* treatment in the biogas reactor. Based on the knowledge about the substrates and the earlier performed studies using enzymes proteases, cellulases, and an α-amylase were assessed. Lysozyme was also selected for the study since it degrades the peptidoglycan in mainly gram positive bacteria and a large part of WAS consists of microbial cell walls, although addition of lysozyme has in earlier studies not been shown to improve biogas production (Nagel et al., 1992). Lipases were however not included in this study since lipase activity has not been much studied earlier, indicating that lipids is not considered to be a major part of sludge at WWTP. Furthermore, lipids are not polymeric substances and it has also been found that release of high concentrations of long chain fatty acids could potentially cause more problems than what is solved (Nordell et al., 2013).

2. Material and methods

2.1. Sludge origin

Waste activated sludge (WAS) and anaerobic digester sludge (ADS) for all experiments were collected at the municipal wastewater treatment plant (Nykvarn WWTP) in Linköping, Sweden. WAS was collected from one of three parallel biological water cleaning steps and the ADS was collected from the first in a series of three anaerobic digesters treating dewatered mixed sludge. The digesters were operated with an average HRT of 20 days at mesophilic conditions (38 °C) and reaches a degree of degradation of approximately 55%.

2.2. Activity and life time of selected enzymes in sludge fluids

In order to evaluate the impact of sludge environment on the activity and life time of added enzymes, the liquid phase of the sludge was used. For both WAS and ADS the material was centrifuged for 10 min at 10 000 × g at 4 °C and the supernatant collected. For ADS, the supernatant was collected and centrifuged in the same way a second time in order to obtain a liquid free of particles. All supernatants were collected and kept on ice prior to the start of experiments for enzyme activity assays (within 4 h). Before assays, pH and conductivity of the supernatants were determined. All assays were performed in duplicates and chemicals were purchased from Sigma Aldrich unless otherwise stated. Enzyme activity and life time was monitored either by absorbance (U-2800A UV-VIS spectrophotometer, Hitachi, Tokyo, Japan) or fluorescence (Fluostar Galaxy, BMG Labtechnologies, Ortenberg, Germany) in freshly prepared liquid fractions of WAS and ADS, respectively.

2.2.1. Cellulase activity assay

For measurement of cellulase activity, cellobioside labeled with resorufin was used (Marker Gene Technologies, Eugene, USA) (Coleman et al., 2007). The assay was performed according to the manufacturer's protocol, by absorbance at 572 nm after 30 min incubation at the same temperature as the digesters were operated at (38 °C). Stock solutions of 50 μM of substrate were prepared in DMSO. Prior to measurement the stock solution was diluted 10 times in 0.1 M sodium acetate buffer pH 6.0. A blank was used in each assay to subtract the background absorbance, either with

substrate and the sodium acetate buffer (for reference sample), or substrate and the respective sludge liquid.

2.2.2. α-amylase activity assay

The α-amylase activity was measured according to the method described by Bernfeld, 1955) and the protocol supplied by Sigma Aldrich. The incubation time was set to 30 min at 38 °C. Starch was used as substrate and the absorbance was recorded at 540 nm and a 20 mM sodium phosphate buffer with 6.7 mM sodium chloride pH 6.9 was used as reference solution.

2.2.3. Protease activity assay

Resorufin labeled casein was purchased from Roche (Mannheim, Germany) and used as substrate for protease activity. The assay was performed according to protocol (Schickaneder et al., 1988), by which the absorbance was measured at 574 nm after 1 h. Reference solutions for modified trypsin was 50 mM sodium acetate buffer pH 7.5 and for subtilisin 10 mM sodium acetate buffer pH 7.5.

2.2.4. Lysozyme activity assay

Cells from *Micrococcus lysodeikticus* labeled with fluorescein was used as substrate for lysozyme activity measurement (Thermo Fischer scientific, Waltham, MA, USA). The assay was performed according to the manufacturers' protocol and the fluorescence was measured at 38 °C with the excitation filter 485 nm and emission filter 520 nm after 30 min incubation at 38 °C.

2.2.5. Activity of endogenous enzymes in sludge fluids

Background enzyme activity from naturally occurring endogenous enzymes in the sludge fluids were measured for all activities in both WAS and ADS liquid. As reference solutions, sludge liquids heated to 100 °C for 10 min, by which endogenous enzymes were expected to be inactivated, were used.

2.2.6. Enzymes

Six different enzymes were selected for the experiment (Table 1). In order to minimize effects on biogas production from organic impurities, residual growth medium or additional activities, highly pure and freeze-dried enzymes were used in all experiments. Cellulase from *Trichoderma viride* (prod. no. C9422), lysozyme from hen egg white (L6876) and α-amylase from *Bacillus licheniformis* (A4551) and two different proteases, subtilisin from *Bacillus licheniformis* (P5380) and trypsin from bovine pancreas (T4665), were all purchased from Sigma Aldrich (Saint Louis, USA). In addition, a modified trypsin (V511), protected against auto proteolysis, was purchased from Promega (Madison, USA).

2.2.7. Activity and life-time of assessed enzymes

Stock solutions of the selected enzymes (1 mg/mL) were prepared (Table 1) in which the freeze-dried enzymes were dissolved according to the following; cellulase in 0.1 M sodium acetate buffer pH 6.0; α-amylase 20 mM sodium phosphate buffer with 6.7 mM sodium chloride pH 6.9; lysozyme in distilled water; subtilisin in 10 mM sodium acetate buffer pH 7.5, trypsin in 1 mM HCl and modified trypsin in 50 mM sodium acetate buffer pH 7.5. Different concentrations of the enzymes were used in the life time experiments depending on the assay sensitivity.

The enzymes were separately added to and diluted in either reference solutions (same as dissolved in), WAS liquid or ADS liquid, respectively. For each sample, 15 mL was prepared to the following final concentrations: cellulase 20 μg/mL, α-amylase 0.25 μg/mL and proteases 0.5 μg/mL. A sample was collected immediately after mixing for activity assay. The remains of each sample were thereafter incubated at 38 °C and the sample volume appropriate for the respective assay was collected over time and the residual activity was measured according to the assays described above.

2.2.8. Activity and life-time in presence of protease inhibitor

In parallel to the activity and life time assays of enzymes added to the sludge liquids, the effect of adding protease inhibitor was investigated for cellulase, lysozyme and α-amylase. Experiments were performed as described in 2.2.7, but with the addition of complete protease inhibitor cocktail (Roche, Basel, Switzerland). A stock solution with the protease inhibitor was prepared in water and thereafter added to the samples to reach the final concentration recommended by the manufacturer.

2.3. SDS-PAGE of cellulase

In an attempt to analyze the proteolysis and fragmentation of added enzyme, a stock solution of 10 mg/mL of cellulase was prepared in 0.1 M sodium acetate buffer pH 6.0. The stock solution was diluted to the final concentration of 1 mg/mL in the sodium acetate buffer, in WAS liquid or ADS liquid, respectively. The samples were incubated at 38 °C and samples were collected at time zero and after 72 h of incubation. Samples and references (liquids with no cellulase added) were run on a Bolt® Bis-Tris gel with a gel concentration of 10% and BenchMark™ was used as molecular size marker (Thermo Fisher Scientific, Waltham, USA). After electrophoresis, the gel was stained with SYPRO Ruby, and an image was captured directly with an ImageQuant LAS4000 (GE Healthcare, Uppsala, Sweden).

Table 1
Properties and biochemical methane potential of BSA and enzymes.

Enzyme/protein	Specificity	pH optimum	Amount added at 1% of VS (mg)	Theoretical BMP of added enzyme per g VS substrate at 1% (NmL)[a]	Increase in biomethane per g VS (for BSA) and calibrated BMP (NmL)[b]
BSA[c]	–	–	87.8	5.39	4.62
Cellulase[c]	1,4-β-D-glucosidic linkage in cellulose	pH 4.2–5.2[e]	87.8	4.80	4.1
α-amylase[c]	1,4-α-D-glucosidic linkage in polysaccharides	pH 7[e]	87.8	5.35	4.6
Lysozyme[d]	Linkages between residues in peptidoglycan	pH 6–9[f]	99.2	5.13	4.4
Subtilisin[d]	Peptide bond	pH 7–10[f]	99.2	5.09	4.4
Trypsin[d]	Peptide bond	pH 7–9[f]	99.2	5.11	4.4

[a] Theoretical BMP of the amount enzyme added at 1% per gram VS substrate, i.e. for 10 mg.
[b] The registered additional biomethane produced from added BSA 24 h after addition (see Fig. 3B). Deviation from theoretical BMP was - 14%. Therefore, the BMP of all enzymes was multiplied by the calibration factor 0.86 in order to compensate for the none-productive presence of water and salts in freeze-dried enzyme preparations.
[c] Batch experiment 1 (450 g anaerobic digester sludge. TS = 3.0%, VS = 65%. Volatile solids = 8.78 g or 19.5 g/L).
[d] Batch experiment 2 (500 g anaerobic digester sludge. TS = 3.2%, VS = 62%. Volatile solids = 9.92 g or 19.8 g/L).
[e] Data from Worthington Biochemical Corporation.
[f] Data from Sigma Aldrich.

2.4. Enzyme effect on biogas production rate and yield in batch experiments

The effect of enzyme addition on the biogas production rate and yield was investigated for complete ADS collected from the anaerobic digester, thus simulating an *in situ* treatment. Experiments were performed in batch with an automatic methane potential test system (AMPTS II, Bioprocess control, Lund, Sweden). The experiments were conducted on two separate occasions with an interval of 8 days, thus sludge was collected twice. Total solids (TS) and volatile solids (VS) of the material was on both occasions determined before addition of enzymes according to Swedish Standard protocol (SS 28113). Digester sludge amount was at the two occasions 500 and 450 g, respectively, and the sludge was distributed in 594 mL glass bottles, pre-flushed with nitrogen.

Experiments were performed for complete digester sludge with each of the enzymes added separately, a reference with no protein/enzyme added for each occasion, and a positive control with a protein with no enzyme activity (protease and volatile fatty acid free bovine serum albumin (BSA), SigmaAldrich, prod. no. A7030). All series were performed in triplicates with an additional fourth glass bottle incubated in a heated cabinet for sample collection. The bottles were sealed according to the manufacturers' instructions and incubated at 38 °C with semi-continuous stirring (1200 s on, and 120 s off) with a stirring speed of 112 rpm for the bottles in the AMPTS II. The fourth bottle for sample collection in each series was incubated at 38 °C on a magnetic stirrer table (RO 15 power, IKA, Staufen, Germany) with semi-continuous (15 min/hour) stirring with a stirrer speed of 300 rpm in the heated cabinet. The biomethane production in the bottles in the triplicate measurement series was continuously logged and approximately 22 h after start up the selected enzyme, or control, was added to each of the respective series. For all enzymes but trypsin, a second enzyme addition was executed when the effect on biomethane production rate from the first enzyme addition had ended. The amount of added enzyme and BSA was in each case 1% (w/w) with respect to the sludge VS and dissolved in 10 mL. This ratio with respect to sludge VS was selected since it was within the range used in earlier studies and further provided an absolute enzyme concentration of approximately 1/5 of the concentration used for the measurements of enzyme activity and life time (or 0.2 mg/mL) and which was judged to be enough for reliable enzyme activity measurements. For the reference series the same volume of water was added to compensate for the extra volume added to the enzyme experiment series. In order to facilitate comparisons, all results were recalculated to represent per gram sludge VS and the addition of 1% enzyme per gram sludge VS.

As a reference of biogas production from the added organic material of the enzymes themselves, the theoretical maximal biochemical methane potential (BMP) from complete anaerobic digestion of each added enzyme was calculated for standard conditions using the Buswell equation (Buswell and Hatfield, 1936) and the ideal gas law. For each enzyme with defined amino acid sequence the elementary composition was calculated using the ProtParam tool in ExPASy (Gasteiger et al., 2005). This is however not the microbially accessible form of the enzymes, which first need to be hydrolyzed for the microorganisms to be able take up the amino acids. Therefore, the Buswell equation was applied to the fully hydrolyzed enzymes by adding one molecule of water for each peptide bond to be hydrolyzed to produce a fully hydrolyzed enzyme. For cellulase, which is a mixture of enzymes rather than a single defined protein, a generic elementary formula of amino acids ($C_{13}H_{25}O_7N_3S$) of proteins was used (Drosg et al., 2013). Furthermore, although all enzymes used were freeze-dried preparations of high purity, not 100% of the mass will be protein but will also be made up by salts and water. To compensate for the none-organic mass of added freeze-dried enzymes, the actual amount of biomethane produced from BSA was compared to the theoretical BMP of BSA to find a calibration factor which was then used for all enzymes (Table 1).

From the fourth bottle, samples were collected at selected time points and the following parameters was monitored: soluble chemical oxygen demand (SCOD), suspended solids (SS), volatile suspended solids (VSS), enzyme activity in the sludge liquid (as described earlier) and volatile fatty acids (VFA). SCOD was determined by Hach-Lange kits according to Swedish standard (SS 28142). The samples were centrifuged for 20 min at 15 550 × g and the SCOD was measured in the supernatant. VFA was analyzed on a gas chromatograph according to a method described earlier (Jonsson and Boren, 2002). The samples were centrifuged for 20 min at 15 550 × g. The supernatant was filtered through a 0.45 μm filter and the samples were stored at −20 °C before analysis. Formic acid was added to the samples and the content of VFA was measured with a gas chromatograph with a flame ionization detector (GC-FID, Clarus 500, Parkin-Elmer, Waltham, USA) and a capillary column with the diameter of 0.32 mm and a length of 30 m was with Helium as carrier gas.

3. Results and discussion

3.1. Environment of enzyme activity and life time assay

Enzyme activity and life time was assayed in the liquid phase of the WAS and ADS, thus representing the environment in which the enzymes are supposed to operate. Therefore the basic parameters that can influence enzyme activity were determined. The pH in the ADS liquid was found to be higher (7.7 ± 0.2) than the pH for WAS liquid (7.0 ± 0.3). These pH values are within the optimal range for the majority of the selected enzymes (Table 1). However, for cellulase, the pH values are significantly higher than the declared pH optimum range. Thus, full activity cannot be expected for cellulase at these conditions. The conductivity measurements resulted in values of 0.26 ± 0.05 mS/cm in the WAS liquid and 8.8 ± 0.1 mS/cm in the ADS liquid. For comparison, this correspond to a NaCl salt concentration of approx. 0.01% and 0.5%, respectively, neither of which can be considered to be an extreme ion concentration.

3.2. Endogenous enzyme activity of sludge fluids

Endogenous cellulase and lysozyme activity were below the detection level in both sludge environments. However, α-amylase activity was determined to 0.020 U/mL in the ADS liquid and 0.015 U/mL in the WAS liquid. More importantly in this context is that the endogenous protease activity could be determined to 0.070 U/ml in ADS liquid and 0.011 U/ml in WAS liquid. That is, the highest activity was registered for proteases and in both cases the higher value was registered in the anaerobic digester environment. It is noteworthy that these results refer to the activity in the liquid phase of the two sludges, free from microorganisms and particulate matter. It is, however, well established that much of the extracellular enzyme activity is associated to the cell wall of microorganisms, as is the case for the cellulolytic activity in e.g. cell wall bound cellulosomes. Furthermore, the endogenous activity has in previous studies been concluded to often be associated to the sludge flocs and solids (Wawrzynczyk et al., 2008) which, together with the enzymes being very dilute in the liquid phase, can explain the low activity observed. Nevertheless, for the purpose of adding enzymes (i.e. catalytic proteins) to these environments, it is interesting to note that the highest detected endogenous activity was from proteases.

3.3. Life time of added enzymes in sludge environments

The collective results of the enzyme activity and life time experiments are summarized in Fig. 1A–F. Three conclusions can immediately be drawn, namely that (*i*) the majority of enzymes are in fact initially active in both the sludge environments, and in most cases approximately to the same degree as in the reference solutions, (*ii*) that all of those enzymes that are initially active in the sludge environments are rapidly inactivated, and in most cases at a faster rate than when incubated in a reference solution and (*iii*) in all cases where protease inhibitor is added (Fig. 1 A–C), the life time of the enzyme is prolonged. There are however some important exceptions.

First, the tested cellulase (Table 1) is not at all active in the digester sludge liquid (Fig. 1A) and apparently this is not due to proteolysis of the cellulases since addition of protease inhibitor does not alleviate this inactivation (lines are superimposed in Fig 1A). The lack of activity can neither be explained by pH since the small pH difference of 0.7 units between the WAS and ADS liquid does not suggest that, and the activity should in any case be higher at lower pH (Table 1), which in this case is in the ADS liquid. Instead, it is more likely that there is an inhibitory substance in the ADS liquid that causes an immediate inactivation of the cellulase in this environment. This is not unreasonable given the large number of metabolites that can be expected to be present in the anaerobic degradation process.

Second, the life time of α-amylase (Fig. 1B) and lysozyme (Fig. 1C) in reference solution was shorter or the same as in the tested sludge liquids (approx. 24 h) and the reason to this is not clear. However, and although delivered as pure enzyme, it cannot be excluded that these freeze-dried enzyme preparations contain low concentrations of proteases. Nevertheless, when protease inhibitor cocktail was added to the sludge liquid samples, the absolute activity of both α-amylase and lysozyme were significantly increased at all equivalent time points when compared to samples without protease inhibitor (Fig. 1A–C).

The activity and life time of the proteases subtilisin and trypsin (Fig. 1 D–E) could for obvious reasons not be examined in presence of a protease inhibitor cocktail and the proteases where in all cases but one inactivated within 12 h. This is to be expected since the

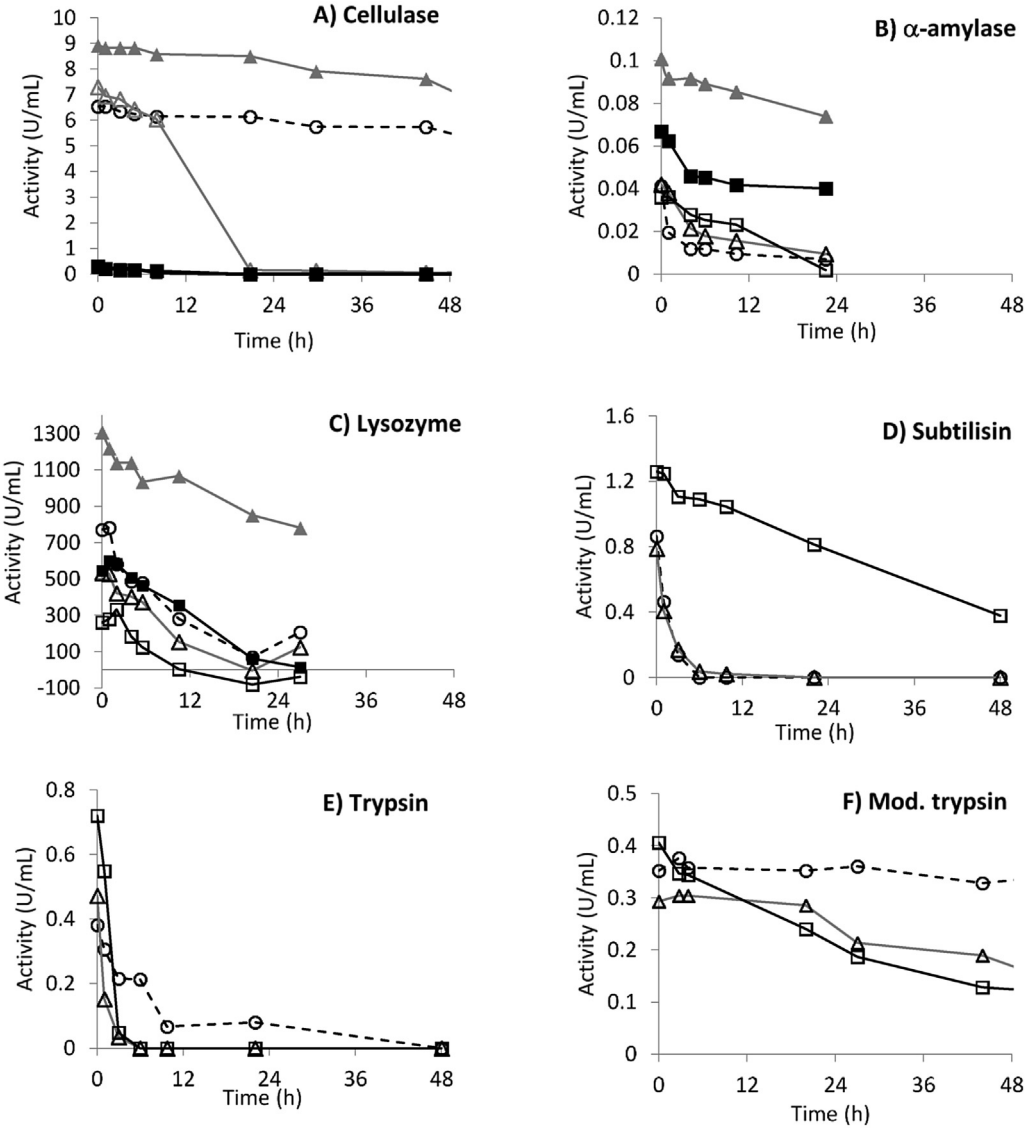

Fig. 1. Activity and life time of enzymes in sludge liquids (legends in figure). In all figures the enzymes are in either: reference solution (- -○- -); WAS liquid (–△–); in WAS liquid with protease inhibitor (–▲–); ADS liquid (–□–); or ADS liquid with protease inhibitor (- -■- -).

enzymes were initially highly active in all liquids (at time zero) and is therefore susceptible to intermolecular autoproteolysis. This autoproteolysis impedes the possibility to discriminate between the inactivation by any possible endogenous proteases in the sludge environment and the autoproteolysis itself. To circumvent this, the experiment was repeated with a chemically modified trypsin (Fig. 1F), protected against autoproteolysis by methylation of lysine. Thus, the enzyme is protected against proteolysis by serine proteases that recognizes substrate protein with a lysine in the P1 position of the serin protease recognition site. For this modified trypsin, the activity life time of the enzyme was almost unaffected in the reference solution during the assay time (Fig. 1F), showing that the enzyme was indeed protected against intermolecular autoproteolysis. However, when analyzed in the respective sludge fluids the activity decreased faster in these environments. This strongly indicates that there is additional protease activity in these environments that inactivates the enzyme, rather than just autoproteolysis by the enzyme itself. Nevertheless, the modified trypsin retained its activity over a longer period of time in sludge liquids when compared to cellulase, α-amylase and lysozyme in WAS liquid and ADS liquid, indicating that the main protease activity in the sludge environments are from serine proteases, to which the modified trypsin is insensitive. Interestingly, subtilisin displays a very different behavior, in reference solution and waste activated sludge environment subtilisin is, similar to trypsin, inactivated very fast. However, in the environment of ADS liquid, subtilisin display a fairly long life time and is not completely inactivated even within 48 h (Fig. 1D). The reason to this behavior is not easily explained, but given that the inactivation rate of subtilisin in digester sludge liquid is approximately the same as for the chemically modified trypsin in the same environment (Fig. 1F) it could be that subtilisin is not at all active in the digester liquid due to environmental reasons, and thus no autoproteolysis occurs and all inactivation is because of endogenous proteases. This monitored behavior could be the result if the enzyme is not active in the sludge, but regains activity in the enzyme activity assay.

3.4. SDS-PAGE analysis of cellulase incubated in digester sludge fluid

The above results, in which the activity and life time was generally higher in a reference solution and was increased in the two sludge liquids by the addition of protease inhibitor for protection against protease activity, indicate to that in most cases the enzyme inactivation is caused by endogenous proteases in the sludge liquids. To verify this, gel electrophoresis of cellulase, the only enzyme to maintain stable high activity in reference solution over time (Fig. 1A), and hence structure, was used to analyze the structural integrity of the enzyme immediately after addition and after 72 h of incubation in the three liquids (Fig. 2). It is apparent that the commercial cellulase preparation contains many proteins but is dominated by a band at approx. 60 kDa which is likely to be the main cellulose degrading enzyme of the preparation.

At time zero, just after addition of cellulase to the different liquids, no significant differences can be observed between the three different solutions. This correlates well with the finding that cellulase in either reference buffer or WAS liquid are both initially active. Furthermore, also for cellulase in digester fluid at least the primary structure is maintained which supports that the inactivation of cellulase in this environment is not due to a rapid degradation of the enzyme but is caused by other factors. After 72 h of incubation the enzyme in reference buffer is unaffected (Fig. 2) which is also noted in the maintained activity of the enzyme (data not shown for this time point). This result further indicates that the cellulase preparation itself does not contain any proteases. In

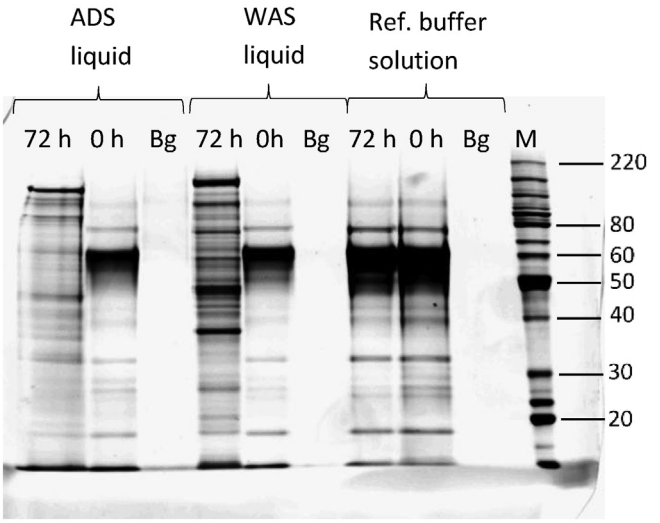

Fig. 2. Electrophoretic separation of cellulase just after addition and after 72 h incubation in ADS liquid, WAS liquid and reference buffer solution (0.1 M sodium acetate, pH 6.0). Bg refers to background, i.e. the respective liquid without cellulase added.

contrast, in neither of the sludge liquids are there anything left of the presumable cellulose in the dominant band at 60 kDa after 72 h of incubation, which is to be expected if the enzyme is inactivated by the degradation by endogenous proteases. Interestingly though, the fragmentation pattern between the two sludge liquids differ, which is likely to be the result of different dominating proteases in WAS and ADS liquid, respectively. Thus, an individual type of enzyme used could be better or worse suited for one or the other sludge environments, depending on the type and number of protease recognition sites in that specific type of enzyme. Furthermore, the fact that distinct fragments can at all be observed after 72 h of incubation is remarkable and suggests that there are a limited number of protease types in action.

A more thorough study of the time dependence of fragmentation could therefore be the basis to understand the cleavage order of added enzymes. Such knowledge could in turn be used to engineer enzymes in which initial and critical protease recognition sites are altered in order to make enzymes that better withstand endogenous proteases in sludge environments. It should be noted that the results also comprise an anomaly since there are some larger fragments present after incubation for 72 h in sludge liquids than in the initial solution (lane 1 and 4 from the left in Fig. 2). These need to be very strongly joined proteinaceous aggregates since they were not dissolved in the sample preparation for SDS-PAGE.

3.5. Biogas batch experiments

From all the above it is obvious that the majority of enzymes is initially active in the two sludge environments but are degraded and inactivated, at different but more or less fast rates. This is however in a purely liquid phase of the WAS and ADS and a different behavior cannot be excluded in presence of surfaces etc. in the form of particles and substrates, which could protect added enzymes from proteolytic degradation to a certain extent. Thus, to test the effect of the enzymes in an envisaged process of adding the enzymes directly to the anaerobic digester, batch experiments were performed. This was judged to be the most applicable scenario since in a real life application it would be better to add the enzyme directly to the higher dry content of an anaerobic digester. In addition, no auxiliary equipment would be needed, as would

otherwise be the case for pretreatment of WAS. Furthermore, besides for cellulase, the above results do not indicate that the efficiency or life time of enzymes would be significantly better in WAS than in the anaerobic digester. For clarity, it should be noted that the experimental set-up is not as in a traditional biochemical BMP test, in which a small inoculum is mixed with a buffer and a fresh substrate in order to find the maximum methane potential of the substrate after complete digestion. Instead, whole sludge collected from the anaerobic digester of the WWTP was used to imitate how, and for how long, an enzyme addition to an anaerobic digester would affect the gas production rate and accumulated gas production during the period of enzyme activity. Therefore, gas production was only monitored over the time period in which there was an effect from enzyme additions as manifested by a difference in biomethane production rate between the enzyme augmented samples and the reference.

Prior to the addition of enzyme, BSA (control) or only water (reference), the ADS was incubated for 22–24 h to allow for easily digested material to be consumed and the batches to equilibrate. Thus, before each addition of enzyme the biogas production flow rate and biogas accumulation were determined to be similar between the series (Fig. 3). Furthermore, by this approach, in which the easily accessible substrate is first allowed to be consumed, the more relevant question of how enzymes affect the degradation of the more recalcitrant part of the sludge is assessed. Directly after addition, the enzyme activities in the supernatant were analyzed in each of the series provided with the selected enzyme. It was found that immediately after addition it was possible to detect the selected enzyme activity in the liquid phase in all series provided with enzyme, except for the already noted inactivated cellulase (Section 3.3). However, already 1 h after addition, the enzyme activity in all series was similar to the reference without added enzymes. This was a significantly shorter time than the determined enzyme activity life time in pure ADS liquid but is most likely an effect of the enzymes adsorbing to the solids and substrates in the batch experiment. A lower activity in the liquid phase compared to the solid phase has also been determined in an earlier study (Wawrzynczyk et al., 2008).

As a point of reference, addition of the enzymatically inactive BSA was found to briefly increase biomethane production rate for approx. 12 h (Fig. 3A), and hence the accumulated biomethane production increased over that period of time (Fig. 3B). This was a fast response, but not unreasonable since this is the effect of degrading a highly accessible, soluble globular protein. The net increase in biomethane production from the protease and VFA free BSA at 1% per g sludge VS after 24 h, and subtraction of the reference value, was 5.60 mL. This value is very close to the theoretical BMP of the added BSA, but deviates by - 14% (Tables 1 and 2). Thus, approx. 14% of the added mass of freeze-dried BSA protein preparation can be estimated to be non-organic material and the theoretical BMP of all enzymes was adjusted accordingly (Tables 1 and 2). Therefore, it is interesting to note that upon addition of cellulase or α-amylase neither the gas production rate, nor the accumulated gas production after 20 h was higher than for the enzymatically inactive albumin. In fact, the accumulated biogas production and net increase of these two enzymes was almost identical to BSA and their respective adjusted theoretical biogas potential (Table 2). This indicates that either these enzymes were completely inactive in the digester environment, as was previously shown for cellulase (Fig. 1A), or there were no cellulose or starch to act upon, of which the latter is more easily digested and therefore possibly not even present in the substrate after the 24 h equilibration period. Interestingly, the shape of the biogas production rate curve for α-amylase differed significantly from the shape of albumin and cellulase in that the peak rate was lower but the relatively higher rate was maintained for a longer period of time (Fig. 3A). However, the amount of accumulated biogas produced was almost the same as for cellulase and BSA (Fig. 3B), indicating that the same amount

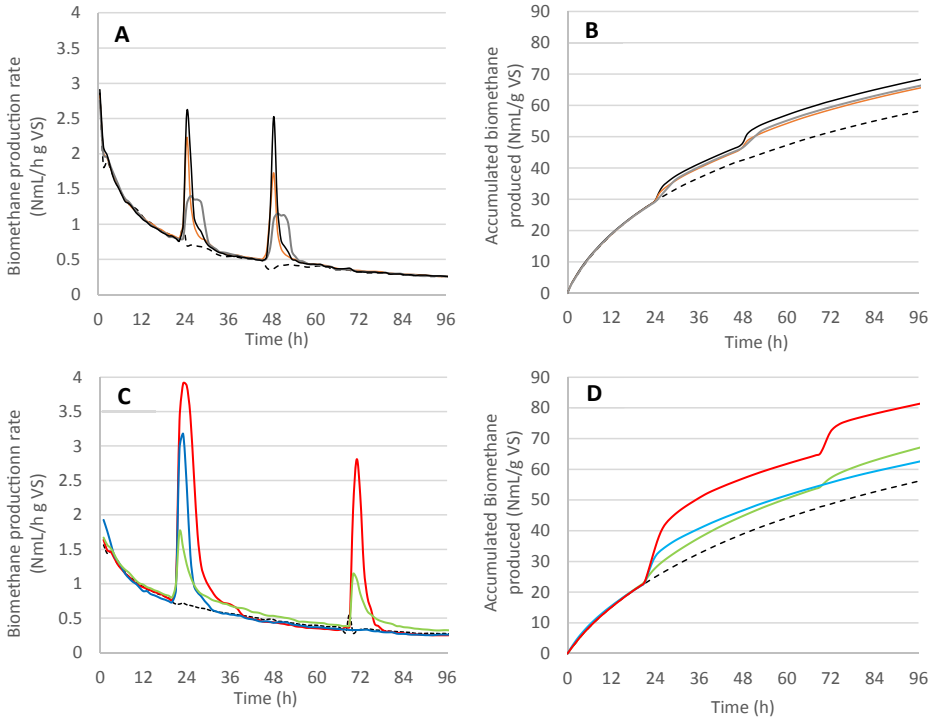

Fig. 3. Results for enzyme batch experiments in anaerobic digester sludge. **A**. Biomethane production rate and **B**. accumulated biomethane produced, upon addition of cellulase (——), α-amylase (——), BSA (positive control) (– – –) and reference with no enzyme/protein added (**- - -**). **C**. Biomethane production rate and **D**. accumulated biomethane produced for subtilisin (——), trypsin (——), lysozyme (——) and reference with no enzyme added (**- - -**). For clarity only four days of monitored gas production is shown.

Table 2
Net effect of enzymes on biogas production.

Enzyme/protein	Net increase at 24 h after addition (NmL/g VS)	Adjusted theoretical potential of added enzyme/protein (NmL/g VS)	Net effect at 24 h after addition (NmL/g VS)	Net percent increase at 24 h after addition[a]
Exp. 1 (450 g)				
BSA	+4.62	4.0	±0	0
Cellulase	+4.3	4.1	0.2	0
α-amylase	+4.5	4.6	−0.1	0
Exp. 2 (500 g)				
Lysozyme[b]	+5.7	4.4	+1.3	4
Trypsin	+7.8	4.4	+3.4	9
Subtilisin	+18.2	4.4	+13.8	37

[a] From absolute values at 24 h after protein/enzyme addition, minus the calibrated theoretical BMP (Table 1), divided by the absolute value of the reference at 24 h after addition (absolute value of references biomethane production per g VS sludge were at these time points 41.8 and 37.5 NmL/g VS for experiment 1 and 2, respectively. See Fig. 3A and B).
[b] Note that lysozyme at this point still had a higher gas production rate than reference, whereas the effect of all other enzymes had ceased (Fig. 4A). Lysozyme reaches almost the net increase of trypsin at 48 h after addition (Fig. 4B).

of α-amylase was added but that α-amylase was more resistant to hydrolysis than the other two enzymes. This supports the notion that different enzymes could display different sensitivity to the endogenous proteases depending on the protease recognition sites of the individual enzyme used (Section 3.4).

The enzymes presenting an effect on biogas production rate and accumulated biogas produced was the proteases and lysozyme (Fig. 3C and D). Of these, subtilisin distinguishes itself by both a very high increase in biogas production rate and a net increase in accumulated biogas produced that is significantly higher than what can be expected from degradation of the enzyme itself (Table 2). This is a much more pronounced effect than for simply adding albumin, and the enzyme therefore most likely contribute to the process by exerting its protease activity by releasing peptides and amino acids. However, the effect on biogas production rate is still transient and approx. 20 h after addition of the enzyme there is a low difference in gas production rate as compared to the reference (Fig. 4A). The reason for this behavior could have two origins, either because all accessible protein had been digested and consumed, or because the enzyme had lost activity. To test for this, a second addition of the same amount of subtilisin was added at approx. 70 h (Fig. 4A and B). Also this second addition of subtilisin resulted in an increase of rate of production and accumulated biogas that was higher than for degradation of the enzyme itself, thus verifying that there was still undigested proteinaceous material present, and further indicating that the ceased effect in the first addition was mainly due to inactivation of subtilisin. Interestingly though, this second addition of the same enzyme to the same amount produced less of an effect, indicating that the first addition had in fact had the desired effect of degrading proteinaceous matter and that there were less protein available to digest in the second addition. This is in contrast to the enzymes not providing a net effect on biogas production rate and yield, by which the first and second addition of enzyme gives the same result because the effect is simply from degradation of the added protein/enzymes themselves (Fig. 3A and B). The finding that subtilisin in fact provides a net effect on biogas production rate and yield is further supported by the notion that after the effect of the first addition of subtilsin has terminated (at approx. 42 h, see red arrow in Fig. 4A and B) the biogas production rate in the subtilisin batch experiment is in fact lower than in the reference. This result, which is also apparent for trypsin, is confirmed by the fact that the accumulated gas production starts to decline slightly, as compared to the reference, from this point forward and until the second addition of enzyme took place (Fig. 4A and B). The above finding, that it is proteases that display a net effect on biogas production rate and yield upon addition to ADS, is further coherent with the findings of low removal efficiency of protein in anaerobic digestion of WAS, by which only approximately one third of the protein is degraded, as compared to half of the carbohydrates after the same time of digestion (Yang et al., 2015). Thus, protein constitutes the largest part of the incoming material and is the least degradable and should consequently constitute a significant part of the residual organic matter in the ADS after anaerobic digestion, on which proteases can act.

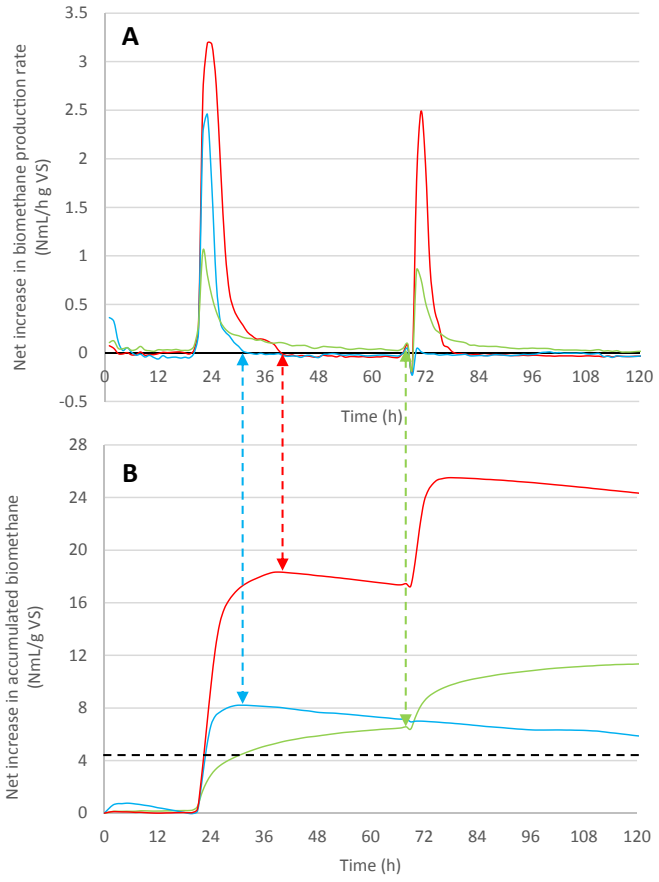

Fig. 4. A. Net increase in biogas production rate and **B**. net accumulated biogas produced upon first and second addition of subtilisin (——) and lysozyme (——) and the single addition of trypsin (——), relative to the reference. That is, in panel A, at a y-axis value of zero there is no difference in gas production rate between the experimental batch bottle and the reference. The broken horizontal line in panel B refers to the amount of biomethane calculated to be produced by anaerobic digestion of the added enzymes. Vertical arrows emphasizes the time at which production rate declines to the same or lower as the reference, and the corresponding net biomethane produced at that point, for the respective enzyme.

Nevertheless, it is problematic that even at a 1% mixture, subtilisin was not able to hydrolyze all available substrate within the limited life time of the enzyme because it means that even higher concentration of subtilisin would be necessary to completely hydrolyze all remaining protein. Trypsin was found to behave almost identically to subtilsin but at a lower level, most probably because of the lower absolute activity observed for the trypsin preparation used (Fig. 1). The above results for the proteases are interesting out of several aspects. First, and most importantly, proteases are evidently active in the digester sludge environment. Second, much of the yet undigested material in the sludge is evidently of proteinaceous nature and there is thus substrate available to increase the hydrolysis rate of. And third, given the fast response in gas production rate and turn-over of the substrate, there appears to be a surplus capacity of the microbial community to handle a higher organic load of microbially accessible nutrients. All of which would be important in a practical implementation of enzymes for increasing the degradation rate of sludge at wastewater treatment plants.

Compared to the proteases, lysozyme has a very different behavior in that the absolute rate enhancement is much lower, but the effect remains for a much longer time. This trailing effect is present for all enzymes (and albumin) but is much more pronounced for lysozyme as it lasts for over 48 h (Fig. 4A and B). The reason to this lies most probably in the action of the enzyme, which act upon the peptidoglycans in the cell wall of bacteria and thereby causes lysis of the cell. This in turn initiates a release of not only monomers and oligomers of a specific substrate, but of the whole cell content of a lysed microbial cell that is then accessible for hydrolysis by endogenous hydrolytic enzymes present in the digester sludge environment. Therefore, and although the life-time of the enzyme itself was short in the digester sludge fluid (<12 h), the effect lasts much longer. In fact, at the time for the second addition of lysozyme the accumulated biogas production in the lysozyme series had reached the same level as for the trypsin series, despite a much lower absolute rate enhancement effect (Fig. 4A and B and Table 2). This is noteworthy since it illustrates how valuable a long life time or effect is because it is the “area under the curve” in the rate enhancement data that is directly translated into more accumulated produced biogas. That is, an enzyme with low rate enhancement and long life time would be just as valuable as a highly efficient enzyme with short life time as it can still produce the same effect in increased accumulated production of biogas. Even more preferably would of course be to employ an enzyme with high enzymatic efficiency with long life time, as this would allow to decrease the enzyme dosage while still retaining the same end effect. It should be noted that although lysozyme does not digest the cell wall of archaea, such as methanogens, the use of lysozyme as an *in situ* treatment in anaerobic digesters could be problematic if excessive cell lysis causes imbalances in the microbial community structure of bacteria susceptible to cell wall degradation by lysozyme. Such high efficiency in cell wall degradation was clearly not reached in the current work, but if more efficient cell lysis could be achieved, lysozyme treatment could instead be used on WAS as a pre-treatment step before anaerobic digestion.

As a complement to biogas production rate and accumulated biogas produced SCOD, SS, VSS and VFA were analyzed prior and after enzyme addition. However, no accumulation could be detected for any of the components regardless of enzyme used. This is likely because whole digester sludge was used, as opposed to a BMP test in which a small inoculum is used. Therefore, the microbial population in our experiments is already from start containing a dense population of all the necessary microorganisms, in correct relative ratio, to handle the sudden increased amount of accessible organic material without the accumulation of intermediate products. This is further evident in the fast response in gas production rate upon addition of proteases.

To summarize the batch experiment, it was found that neither cellulase nor α-amylase had any effect on gas production rate or net increase in produced biogas beyond the BMP of the added enzyme itself. Lysozyme and trypsin had a small effect on rate and biogas yield, however, only approximately doubled the effect from the biogas potential of the enzymes themselves (Fig. 4B). For lysozyme this effect was reached approx. 48 h after addition (Fig. 4B and Table 2). The only enzyme that produced a significant effect on biogas production rate and yield was subtilisin with a net effect approximately three times higher than the BMP of the enzyme itself. This corresponds to a net increase in accumulated biogas production of 37% after 24 h from addition (Table 2). At first sight this would appear to be an impressive effect, however, it should be noted that this is the percent increase from a low level of gas production from the recalcitrant residual part of the digester sludge. Recalculated to one metric ton of digester sludge with the same amount of VS and same relative amount of added subtilisin (1% of VS) this corresponds to a need of 198 g of freeze-dried subtilisin product to increase the biomethane production by 0.275 Nm^3/ton sludge (up from 0.742 to 1.017 Nm^3/ton sludge), and it is questionable if the value of the extra 275 L biomethane produced can cover the cost even of a technical grade subtilisin to reach this increase. Still, it cannot be denied that subtilisin has an effect, and that the main problem is the short life-time of the enzyme in the sludge environment. Thus, if the life-time of the enzyme could be substantially increased, or alternative enzymes better suited to the environment of anaerobic digesters could be identified (Speda, J. et al., 2016), much less enzyme would be needed and the use of enzymes to increase biogas production and yield could possibly be reached.

4. Conclusions

Almost all of the selected hydrolytic enzymes were initially active in both of the sludge environments. The activity life time was, however, limited for all the enzymes in both environments due to degradation by the activity of endogenous proteases. A net increase in biogas production rate and yield could still be observed upon addition of both proteases and lysozyme. However, even in the best case the cost/benefit ratio is likely too high at the enzyme concentration needed to reach this effect. Thus, enzymes with higher activity and/or longer life time, enabling lower dosages to reach the same effect is desirable for implementation.

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