Water Research 103 (2016) 462–471

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

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

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ARTICLE INFO

*Article history:*
Received 15 April 2016
Received in revised form
13 July 2016
Accepted 26 July 2016
Available online 29 July 2016

*Keywords:*
Wastewater treatment plant
Biogas
Enzyme
Protease
Subtilisin

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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http://dx.doi.org/10.1016/j.watres.2016.07.064
0043-1354/

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