Intensification of anaerobic fermentation from proteinaceous industrial wastes by adapted microbes

Ph.D. Thesis

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Introduction

The energy consumption of our welfare society is rapidly increasing and at the same time environmental pollution and concomitant climate deterioration is reaching its crucial stage. The energy consumption of the world is currently around 220 EJ per a year (DOE/EIA-0484, 2013). 78% of this energy demand is covered by fossil energy carriers. The depletion of economically recoverable resources occurs in the foreseeable future therefore we need to look for new opportunities (Energia a Napból, 2013). Renewable energy is expected to increase nearly 50% in the next 35 years, however, according to the predictions biomass-derived energy will contribute with only 3 EJ/year (DOE/EIA-0484, 2013). Energy plants and the use of plant-based materials is dominating the energetic use of biomass. They do not reduce net CO_2 emission into the atmosphere, but at least their energetic conversion does not increase the amount of carbon in circulation (Reményi, 2007). In the last twenty years carbon dioxide emissions significantly decreased although the reduction reaches only 6% relative to the reference years, (Reményi, 2007). The European Union decided on a compulsory reduction of greenhouse gas emission by 20% before the year 2020 and 20% of the produced energy has to derive from renewable sources (Federal waste management plan, 2006). Hungary assumed 14.65% reduction, currently this value is around 7%.

Anaerobic degradation and the accompanying biogas production is an especially appealing way of disposing of the organic waste and conserve the environment at the same time. Biogas production can help solving some environmental problems, e.g. waste management, reduction of pollution and carbon-neutral renewable power generation. Furthermore it can help to return nutrients to the soil replacing in part the energy demanding artificial fertilizers (Tafdrup, 1995). Raw biogas, after cleaning and enrichment, offers many opportunities for use, e.g. heating, conversion in gas engines to electricity and heat or, after additional CO_2 removal, the obtained biomethane we can injected into the natural gas network for storage and transportation to be used as vehicle fuel or in any other application natural gas is utilized today. Despite the industrial-economic importance of the underlying microbiological events, little is known about the roles and activities of the microorganisms which inhabit the anaerobic niches. In the European Union, more than 8,000 biogas plants are currently operating, but none of them process primarily protein-rich waste, despite huge amount of such materials being generated continuously. These pollutants mount up as meat consumption increases. The roughly 1 million tons of protein-rich waste produced annually by pig, cattle and bird breeding worldwide contains manure, blood and feathers, a biomass type classified as hazardous waste

in several countries. This means 500,000 tons of protein-rich waste per year in the EU alone. Many other parts of the world faces similar problem.

Proteins are composed of amino acids linked by peptide bonds, which are hydrolyzed by proteases upon decomposition. Amino acids are fermented via different pathways, depending on the nature and concentrations of the amino acids present. The degradation products include short- or branched-chain organic acids, NH_3, CO_2, H_2S and H_2. Amino acids are metabolized through two main routes: pairs of amino acids can be decomposed through the Stickland reaction; and single amino acids can be degraded in the presence of H_2-utilizing bacteria. The Stickland reaction is the simplest way to degrade amino acids and provides the cell with 0.5 mole of ATP per mole of amino acid transformed. AD demands the concerted action of many groups of microbes, each performing their special role in the overall degradation process.

The optimal carbon/nitrogen/phosphorus (C/N/P) ratio for a high methane yield is around 100:3:1. The digestibility of carbohydrate-rich wastes can be improved by mixing them with substrates of high nitrogen content, thereby improving the C/N ratio. In anaerobic fermentation, the acidogens and methanogens differ in their physiology, nutritional needs, growth kinetics and sensitivity to the environmental conditions. Failure to sustain the balance between these two groups is the main cause of process instability. The introduction of energy-rich proteinaceous waste products in large quantities into the anaerobic degradation process is not recommended in view of the increased risk of inhibition by ammonia. In the literature, the inhibitory level of the total ammonia concentration varies, depending on conditions such as the inoculum, the substrate, the acclimation need, the operation period, pH and temperature. Free NH_3 is the main cause of inhibition since it is membrane-permeable and it causes a proton imbalance and/or a potassium deficiency. The ammonium ion (NH_4^+) is less toxic.

Objectives

Recognizing the huge amount of proteinaceous agricultural and industrial by-products awaiting proper disposal and their enormous biogas potential, the following tasks for this Thesis work were as follows.

1. Development of an adaptation strategy, which brings about the restruring of the microbial community.
2. Application of the adaptation strategy to successfully and efficiently decompose various protein-rich substrates as monosubstrates.
3. Examination of the alterations in the composition of the microbial community during adaptation to proteinaceous substrates with metagenomic analysis using next generation DNA sequencing technique.
4. Addition of pure cultures of carefully selected strains identified with the help of the results of the metagenome analysis to the non-adopted system and test their beneficial effects on the conversion of protein-rich substrates to biogas.
5. Establishment the suitability and efficiency of the adopted microbial community for sustainable biogas production for extended period of time and determination of the possibility to scale-up the process.

Methods

The adaptation experiments were carried out in various anaerobic fermenter arrangements. 0.5 litre batch fermenters and 5-litre volume continuously stirred tank reactors, the latter both in fed-batch and continuous operational modes. Protease enzyme activity measurements were done using dye-labelled casein substrate in a spectrophotometric assay. The new generation SOLiD DNA metagenomic sequencing technique was employed to determine the taxonomic distribution and relative abundance of the members of the microbial community. Quantitative polymerase chain reaction was employed to monitor the bacteria added to the system. In addition several operational parameters were observed. The changes in ammonium-nitrogen concentrations were followed during the fermentations. Volatile fatty acid compositions (acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic acids) were determined using HPLC to monitor the microbiological activity in the reactors. Total carbon and total organic carbon contents have been examined to establish the C/N ratio of the biomass.

Volumetric biogas yields gave information about the efficacy of the anaerobic digestion process. The composition of the evolved gas was measured by gas chromatography.

Results

1. A methodological strategy was worked out and tested on several protein-rich substrates, which can boost biogas production and adapt the anaerobic microbial community to a high protein and a high nitrogen tolerance. Microbes are able to adjust their metabolism to relatively high ammonia concentrations as expected from some previous literature reports, others contradicted these findings. The adaptation strategy invented in our system was based on the protease activity of the microbes acclimatizing themselves to the unusual monosubstrate.

2. The results indicate that after an appropriate adaptation period the biogas production system may become familiar with this substrate and degrades protein-rich substrates, i.e. pig blood, casein, meat extract or vegetarian kitchen waste can be fed into the biogas reactor as monosubstrate. Our results overthrow the dogma of the biogas industry, i.e. in the reactors performing anaerobic degradation of organic matter substrates must be employed at about C/N ratio of 20-30.

3. With the help of metagenome sequencing it was demonstrated that major changes occurred in the microbial composition during the adaptation to protein feedstock. Our data gained by new generation DNA sequencing provided new information about the microbial communities habituating the biogas reactors fed with proteinaceous substrates. Some of our results are in good agreement with those of similar studies. In general, we could establish that a community with similar composition was developed in biogas fermenters digesting various protein-rich substrates. These observations contribute to the design of biogas production microbial communities specialized to this class of previously unused substrates.

4. Based on the results from the metagenome analysis *Bacillus coagulans, Bacillus subtilis and Pseudomonas fluorescens* strains were chosen and their pure cultures were added to the fermenters digesting meat extract for biogas production. The mixture of selected bacteria achieved a 50% increase in methane production without any preliminary adaptation when the system was switched from the mixed substrate of pig manure and maize silage to the protein-

rich one. This proved that it is possible to produce increased yield of biogas with the help of appropriately assembled microbial consortium in spite of a sudden change in substrate composition, which otherwise usually pose serious problems, i.g. failure of the effective anaerobic degradation, in biogas fermenters. The fate and survival of the added bacteria was monitored by quantitative PCR. Following a single inoculation their abundance reached a steady-state level and remained stable for the 3 months duration of the experiment. The elevated methane production from meat extract was sustained during this period.

5. In separate studies the sustainability of the biogas production from protein-rich monosubstrate was tested. The fed-batch system successfully carried out stable biogas generation in the long-term experiment. The specific biogas production rate did not change upon 10-fold volumetric increase, which demonstrated the possibility to scale-up the process.

Publications

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