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Augmented biogas production from protein-rich substrates and associated metagenomic changes

Etelka Kovács [a,b], Roland Wirth [a], Gergely Maróti [b], Zoltán Bagi [a], Katalin Nagy [c], János Minárovits [c], Gábor Rákhely [a,d], Kornél L. Kovács [a,c,d,*]

[a] Department of Biotechnology, University of Szeged, H-6726 Szeged, Közép fasor 52, Hungary
[b] Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, H-6726 Szeged, Temesvári krt. 52, Hungary
[c] Department of Oral Biology and Experimental Dental Research, University of Szeged, H-6720 Szeged, Tisza L. krt. 64, Hungary
[d] Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, H-6726 Szeged, Temesvári krt. 52, Hungary

HIGHLIGHTS

- After appropriate acclimation, sustainable biogas production is achievable from low C/N substrates.
- Metagenomic analysis reveals alterations in the microbial community.
- High biogas yields in anaerobic degradation (AD) of protein-rich monosubstrates.
- Addition of selected protein-degrading strains leads to effective AD.
- Selected strains lead to sustainable AD without acclimation.

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ABSTRACT

This study demonstrates that appropriate adaptation of the microbial community to protein-rich biomass can lead to sustainable biogas production. The process of acclimation to these unusual mono-substrates was controlled by the protease activity of the microbial community. Meat extract (C/N = 3.32) and kitchen waste (C/N = 12.43) were used as biogas substrates. Metagenome analysis highlighted several mesophilic strains that displayed a preference for protein degradation. *Bacillus coagulans*, *Bacillus subtilis* and *Pseudomonas fluorescens* were chosen for detailed investigation. Pure cultures were added to biogas reactors fed solely with protein-rich substrates. The bioaugmentation resulted in a 50% increase in CH_4 production even without any acclimation. The survival and biological activity of the added bacteria were followed in fed-batch fermenters by qPCR. Stable biogas production was observed for an extended period of time in laboratory CSTR reactors fed with biomass of low C/N.

1. Introduction

The consumption of energy is rapidly increasing, and the related environmental pollution and climate changes are approaching critical stage requiring sustainable solutions. The human population worldwide uses around 220 EJ per year (US-EIA, 2013), 78% of this being supplied by fossil energy carriers. The virtual depletion of economically recoverable fossil resources is predicted in the foreseeable future, and the search for new opportunities is therefore warranted. Renewable energy production is expected to increase by nearly 50% in the next 35 years, whereas it is predicted that biomass-derived energy will contribute only 3 EJ yr^{-1} (US-EIA, 2013). Biomass plants and the use of green biomass are currently attracting increased interest in view of their CO_2-neutral nature (Tafdrup, 1995), these technologies therefore not contributing to global environmental changes.

The European Union (EU) has earmarked a joint effort to reduce the emissions of greenhouse gases by 20% and 20% of the energy produced should be supplied from renewable sources by 2020 (Directive, 2009).

Anaerobic degradation (AD) and the accompanying biogas production is a particularly attractive way to generate renewable energy, as the disposal of organic waste from various sources is

* Corresponding author at: Department of Biotechnology, University of Szeged, H-6726 Szeged, Közép fasor 52, Hungary.
E-mail addresses: kovacse@brc.hu (E. Kovács), roland.w@freemail.hu (R. Wirth), maroti.gergely@brc.mta.hu (G. Maróti), bagiz@brc.hu (Z. Bagi), stoma@stoma.szote.u-szeged.hu (K. Nagy), minarovits.janos@stoma.szote.u-szeged.hu (J. Minárovits), rakhely@brc.hu (G. Rákhely), Kovacs.kornel@brc.mta.hu (K.L. Kovács).

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associated with energy carrier production. In addition to waste management and carbon-neutral renewable power generation, the technology helps return essential nutrients to the soil (Tafdrup, 1995).

After cleaning, biogas offers various opportunities for use as an energy carrier, as bio-CH_4 it becomes equivalent to fossil natural gas and the two CH_4 streams are interchangeable in all applications.

More than 12,000 biogas plants are currently operating in the EU, but none of them process primarily protein-rich waste, despite huge amount of such materials being generated continuously.

At least 30% of the weight of animals processed for food ends up as slaughter-house protein-rich waste. The world's annual meat production is around 300 million t, topped up with some 180 million t of milk production, which yields well over 100 million t of protein-rich waste produced annually by pig, cattle, sheep, fish and bird breeding worldwide (FAO Statistics Division, 2009), and a significant proportion of this type of biomass is classified as hazardous waste in many countries. This protein-rich biomass would be extremely valuable as biogas substrate if the basic questions concerning its AD could be solved. Besides the advantages of the biogas produced, the environmentally harmless fermentation residue is an excellent fertilizer for agriculture.

Proteins are decomposed through hydrolysis by proteases. Amino acids are metabolized via 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 (Ramsay and Pullammanappallil, 2001). Stickland reactions are usually faster than uncoupled amino acid fermentation (Ramsay and Pullammanappallil, 2001).

AD involves the concerted action of many microbes from various taxonomic groups, each performing a special role in the overall degradation process (Ahring, 2003; Wirth et al., 2012; Kovács et al., 2014). CH_4 formation is influenced by a number of environmental factors, including the NH_3 concentration (van Velsen, 1979). In AD, NH_3 is produced through degradation of the N-containing biomass present in the feedstock, mostly in the form of proteins (Kayhanian et al., 1999). NH_3 can enter cells in the unprotonated form, which readily diffuses across the cell membrane, equilibrating the intracellular and extracellular NH_3 concentrations. On the other hand, the NH_4^+ ion does not readily diffuse through lipid membranes (Kleiner, 1993). At least two possible mechanisms of NH_3 toxicity have been postulated: (i) NH_3 could inhibit the activity of cytosolic enzymes directly, or (ii) NH_4^+ accumulated inside cells might be toxic through its effect on the intracellular pH (Sprott et al., 1984) or on the concentrations of other cations such as K^+ (Sprott and Patel, 1986). Ca^{2+} or Mg^{2+} triggers antagonistic effects to NH_4^+. In the absence of terminal electron acceptors such as NO_3^-, O_2 or SO_4^{2-}, the methanogenic conversion of organic matter is an essential feature of many ecosystems (Conrad et al., 1989). The optimal carbon/nitrogen/phosphorus (C/N/P) ratio for a high CH_4 yield is around 100:3:1 (Conrad et al., 1989). In AD, the acidogens and methanogens differ in their physiology, nutritional needs, growth kinetics and sensitivities to the environmental conditions (Kayhanian, 1994). Failure to sustain the balance between these two groups is the main cause of process instability (Demirel and Yenigun, 2002).

The introduction of energy-rich proteinaceous waste products in large quantities into the AD process is not recommended in view of the increased risk of inhibition by NH_3 (Ahring, 2003). The quantity of NH_3 generated from AD of an organic substrate can be estimated (Nielsen and Angelidaki, 2008). The fraction of NH_3 relative to the total (NH_3 + NH_4^+)-nitrogen (TAN) is dependent on pH and temperature (Hansen et al., 1996). Inhibitory thresholds for NH_3 have been reported in a number of studies, but the limiting concentrations varied significantly (Kroeker et al., 1979; Angelidaki and Ahring, 1993; Banks and Wang, 1999, Hansen et al., 1996).van Velsen (1979) concluded that a TAN between 0.2 and 1.5 g L^{-1} has no effect on CH_4-forming microbes. Koster and Lettinga (1984) reported that the maximum methanogenic activity under mesophilic conditions was unaffected at a TAN concentration of 0.68 g L^{-1}. However, a TAN concentration between 1.5 and 3 g L^{-1} was found to be inhibitory at pH > 7.4 (van Velsen, 1979). A TAN concentration of 4 g L^{-1} or more inhibited thermophilic digestion of cattle manure (Angelidaki and Ahring, 1993). The inhibitory TAN concentrations mentioned in different studies are seldom comparable, unless the pH and temperature conditions are also stated.

Microbial adaptation to higher NH_3 concentrations and cation antagonism effects (Chen et al., 2008) may also contribute to the broad range of inhibitory NH_3 concentration thresholds reported in the literature. Higher tolerances can be achieved by acclimation and NH_3-adapted anaerobic consortia were observed to be inhibited at an NH_3 concentration of 0.7–1.1 g NH_3-N L^{-1} (Angelidaki and Ahring, 1993, Hansen et al., 1996). Hashimoto (1986) demonstrated that NH_3 inhibition began at about TAN concentrations of 2.5 g L^{-1} and 4 g N L^{-1} for unacclimated and acclimated thermophilic methanogens, respectively. A tolerance of up to 3–4 g NH_4^+-N L^{-1} for an adapted process has also been described (Angelidaki and Ahring, 1993).

Edström et al. (2003) used blood, stomach and intestinal content and food waste in co-fermentation with animal manure. Feedstock mixtures containing 8–15% of animal waste products could be co-digested under stable conditions at a total concentration of 4.5–5.0 g NH_4^+-N L^{-1}.

It is generally acknowledged that, of all the microorganisms involved in AD, the methanogens are the least tolerant to inhibitors and the most likely to cease growth due to NH_3 inhibition (Kayhanian, 1994). Elevated NH_3 levels cause changes in microbial communities; the shift from aceticlastic methanogenesis to syntrophic acetate oxidation is a consequence of the effect of the inhibition by NH_3 (Westerholm et al., 2012). Tests of NH_3 toxicity on the acetate- and hydrogen-utilizing populations reveal a higher sensitivity of the aceticlastic relative to the hydrogenotrophic methanogens; the specific growth rate for the aceticlastic methanogens was halved at an NH_3 concentration of 3.5 g N L^{-1}, as compared with 7 g N L^{-1} for the hydrogenotrophic methanogens (Angelidaki and Ahring, 1993). A lower biogas yield and/or CH_4 yield was observed, however, in the case of an elevated NH_3 load (Koster and Lettinga, 1988).

A common feature of NH_3 adaptation attempts was that the substrate with a high N content was fed together with a substantial amount of C-rich materials in order to approach the recommended C/N/P ratio.

The goal of the present study was to extend the C/N range of protein-rich materials suitable for effective and sustainable AD and biogas production following a controlled adaptation of the microbial community to the unusual substrates. In addition the possibility to eliminate the adaptation period by adding selected protein degrading microbes in pure culture was tested. These bacteria were expected to help the community to cope with the stressful task of utilizing the protein-rich substrates. The practical implications of this strategy for industrial scale biogas production are evident.

2. Methods

2.1. Substrates, inoculum and bacterial strains

Meat extract was purchased from Sigma, and vegetable-rich kitchen waste was made by mixing various fruits (apples, melons, pears and plums) and vegetables (potatoes and tomatoes) in a blender, and the homogenized material was stored in aliquots at −20 °C. The parameters of the biogas substrates are to be seen in

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