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Identity and diversity of archaeal communities during anaerobic co-digestion of chicken feathers and other animal wastes

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ABSTRACT

Digestion of raw feathers in anaerobic digesters inoculated with adapted swine manure, slaughterhouse sludge or dairy manure was investigated using twelve 42-L anaerobic digesters at 25 °C. After 120 days 74%, 49% and 40% added feathers were converted to methane in swine manure, dairy manure and slaughterhouse sludge anaerobic digesters respectively. 16S rRNA gene clone library analyses identified twenty-one operational taxonomic units containing clone sequences from 5 genera, 5 families and 2 phyla of members of the *Archaea* from 158 sequenced clones. Fluorescence *in situ* hybridization revealed that methanogens from the *Methanomicrobiales*, *Methanosarcinales* and *Methanobacteriales* constituted a major fraction (>78%) of these *Archaea*. A high correlation was seen between the distribution of functional archaeal groups and the NH_3–N levels of digester mixed liquors. The compositions of archaeal communities fed different substrates were statistically significantly different ($P < 0.05$).

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1. Introduction

Feathers are a major by-product of the poultry industry, composed of >90% keratin, mainly as β-keratin (Astbury and Beighton, 1961). β-Keratin contains extensive cross-linked disulfide bonds making feathers highly recalcitrant to biological degradation in their natural state (Brandelli et al., 2010). Millions of tonnes of feathers are generated annually and so their disposal is an important part of solid waste management (Onifade et al., 1998). Feathers are usually incinerated or used as landfill, but both are wasteful because their keratin is rich in useful amino acids. Moreover, disposal by such methods generates considerable amounts of greenhouse gases. Recent legislation has restricted the disposal of organic residues into landfill, while other disposal costs have been rising (Korniłłowicz-Kowalska and Bohacz, 2011). Feathers can be recycled after cooking at high pressures and temperatures to generate a meal suitable for use as an animal protein supplement (Odetallah et al., 2003). However, legislation covering the use of organic materials as animal feeds is becoming tighter in attempts to decrease the risk of disease transmission (Korniłłowicz-Kowalska and Bohacz, 2011).

Anaerobic digestion offers an attractive alternative for processing feathers into valuable products, including methane, a combustible fuel, and generating a digested material rich in NH_3 nitrogen (NH_3–N) with a potential use in agriculture as a nitrogen fertilizer. Such a process consumes comparatively little energy, and is environmentally safe (Onifade et al., 1998). However, feathers and other poultry wastes are notoriously difficult to degrade under anaerobic conditions (Korniłłowicz-Kowalska and Bohacz, 2011). Moreover, anaerobic feather digestion remains a challenge since NH_3–N generated from feather keratin digestion is one of the most common toxic substances encountered during anaerobic digestion (Chen et al., 2008). NH_3–N induced inhibition has been reported over a wide range of NH_3–N concentrations (1.7–14 g L^{-1}) during the anaerobic digestion of swine manure, slaughterhouse sludge and dairy manure (Chen et al., 2008).

No studies yet have investigated raw feather digestion with animal wastes other than poultry wastes (Williams and Shih, 1989). Consequently, whether such processes also suffer from NH_3–N inhibition is not clear. We found in our previous study that keratin hydrolyzing organisms (KHOs) in our anaerobic digesters fed with swine manure plus feathers were members of *Alkaliphilus* in family *Clostridiaceae* and the abundance of KHOs inside of feather bags was correlated to the feather degradation rate (Xia et al., 2011). However, knowledge of the chemical transformations involved in feather degradation and the structure and function of the archaeal communities involved in such systems is still lacking. Archaeal

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methanogens are responsible for the terminal steps in methane formation. Their distinguishing characteristics including low growth rates, a high susceptibility to external conditions, and a limited substrate utilization spectrum make such anaerobic digestion processes fragile and highly sensitive to environmental influences (Hori et al., 2006). Thus, it is important to elucidate which archaeal populations are capable of tolerating any inhibitory effects from NH_3–N after a period of acclimation and long-term adaptation (Karakashev et al., 2006), and how different substrates affect the composition and diversity of their communities.

In this study, ground raw feathers were added to digesters inoculated either with adapted swine manure (SM), slaughterhouse sludge (SS) or dairy manure (DM). The digestion of these feathers was monitored chemically and the levels of methane generated were quantified. The composition and biodiversity of archaeal communities developing in these digesters were investigated using full-cycle rRNA approach (Amann et al., 1995). Clone libraries were constructed for archaeal 16S rRNA genes, and fluorescence *in situ* hybridization (FISH) probes with perfect matches to the target sites in selected 16S rRNA clones were applied or if necessary designed to identify and quantify *in situ* the major archaeal groups, allowing the composition and diversity of the archaeal communities in these digesters to be described for the first time.

2. Methods

2.1. Source and preparation of chicken feathers

Freshly plucked white chicken feathers were collected from a slaughterhouse (Saint-Anselme, QC, Canada) and transferred to the laboratory within 4 h. They were divided into 2 kg portions, placed in clean cotton bags and washed (delicate cycle) in a washing machine (Frigidaire, Martinez, GA, USA) using tap water, before being dried at 45 °C in a Unithern dryer box (Construction CQLTD, England) until a constant weight was reached (after about 8 weeks). Each sample was ground through a 4-mm screen (Thomas-Wiley Laboratory Mill) and divided into portions of ≈33 g each and placed in a nitrogen-free polyester bag with a pore size of 50 μm (ANKOM Technology, Macedon, NY, USA). Each bag was sealed with a plastic tie wrap and washed again as above to remove any remaining residual particles <50 μm. Finally, the bags were dried at 45 °C until a constant weight (≈31 g each) was achieved.

2.2. Anaerobic digester setup

Three 7-m^3 semi-industrial scale digesters (Xia et al., 2011) were used to stabilise fresh SM collected from a commercial pig farm (Sherbrooke, Quebec), SS obtained from a commercial cattle slaughterhouse (Colbex, Quebec) and DM collected from a commercial dairy farm (Sherbrooke, Quebec), and each was used as inoculum for twelve 42-L Plexiglas lab-scale digesters described by Xia et al. (2011). When these inocula were added, all these digesters had been operating at 25 °C for >2 years with a retention time 14 days. Four 42-L digesters (duplicate experimental reactors) were each fed initially with 35-L of either adapted SM, SS or DM respectively. Two (duplicate digesters) were added with feathers and another two (duplicate digesters) were used as control digesters without feather addition. Twelve feather bags representing 25.8%, 38.7% and 24.3% of the total chemical oxygen demand (COD) loading ratio were added to each of the SM (SMDs), SS (SSDs) and DM digesters (DMDs) respectively at day 0. All digesters were operated in a temperature controlled room at 25 °C for 120 days.

2.3. Physiochemical characterization of anaerobic digesters

Total solids and total suspended solids were determined according to standard methods (APHA, 1998). Total COD and soluble COD were determined according to the closed reflux colorimetric method described in the standard methods (APHA, 1998). Methane, total Kjeldahl nitrogen, NH_3–N and volatile fatty acids (VFAs) were analyzed following the procedures described by Xia et al. (2011). Physiochemical characteristics of the raw feathers used in this study were determined as detailed by Schrooyen et al. (2000). At day 120, all feather bags were removed, washed with deionized water, dried at 45 °C for 48 h, and weighed to determine the extent of their biodegradation.

2.4. Methanogenesis of feathers and statistical analysis

The methanogenesis in the digesters to which the feathers had been added was estimated using CH_4 production. The CH_4 data and initial feather masses were converted to units (grams) of COD equivalents. The level of methanogenesis from feather digestion was estimated according to the following equation modified from O'Sullivan et al. (2006):

$$\text{Methanogenesis\%} = (\text{COD}_{\text{CH}_4\text{feather}} - \text{COD}_{\text{CH}_4\text{Control})} / \text{COD}_{\text{initial}} * 100\%$$

Where $COD_{initial}$ is the feather COD initially added to a digester, $SCOD_{CH_4feather}$ is the amount of CH_4 COD produced from the feather digesters and SCODCH4Control is the amount of CH_4 COD produced from the corresponding control digesters. Statistical analyses were performed using StatSoft Statistica version 8.0. All reported values were the average of observations made over a 120-day period. Gas production values were daily averages ($n = 120$) while gas composition/VFA/ NH_3–N values were averaged over 6-day cycles ($n = 20$). The level of significance was set at $P < 0.05$.

2.5. Clone library analyses and sequencing

Every 40 days fresh mixed liquor samples taken from a digester were transferred in 2 ml eppendorf tubes that were immediately frozen in liquid nitrogen and stored at −80 °C for later DNA extraction. Mixed liquor samples were also fixed in ice-cold paraformaldehyde (PFA) (4% final concentration) (Amann, 1995) for FISH probing of *Archaea* (see below). Total genomic DNA was extracted from samples stored at −80 °C in triplicate with a FastPrep®-24 System & Kit following the protocol provided by the supplier (MP Biomedicals, OH 44139, United States), after sample pre-treatment with enzyme cocktails (Kong et al., 2010). DNA from samples taken at day 40, 80 and 120 from each digester was pooled in equal mole amounts before being pooled again with DNA extracts from their corresponding duplicate digester, and used as PCR templates. The archaeal universal primers ARC-8F (5′-TCCGGTTGATCCTGCC-3′) and ARC-1492R (5′-GGCTACCTTGTTACGACTT-3′) (Teske et al., 2002) were used to PCR amplify 16S rRNA genes as follows: 30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 55 °C), and extension (2 min at 72 °C) before a final extension at 72 °C for 8 min. The correct sizes of PCR amplicons were checked by screening on 1% agarose gels, and purified with a QIAquick PCR purification kit (Invitrogen, Ontario, Canada) according to manufacturer's instructions, before being ligated into the pCRII-TOPO vector provided in the TOPO TA cloning kit (Invitrogen, Ontario, Canada). Clones with the correct sized inserts were sequenced with primer pair M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) at Laval University (http://www.bioinfo.ulaval.ca/seq) with an ABI 3130xl Genetic Analyzer (Applied Biosystems-Hitachi, Foster City, CA).

2.6. Phylogenetic and biodiversity analyses

Partial 16S rRNA gene sequences were trimmed and assembled using Sequencher 4.5 (Gene Code Cooperation) before being

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