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Anaerobic digestibility of beef hooves with swine manure or slaughterhouse sludge

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ABSTRACT

Anaerobic digestion is an effective method for treating animal by-products, generating at the same time green energy as methane (CH_4). However, the methods and mechanisms involved in anaerobic digestion of α-keratin wastes like hair, nails, horns and hooves are still not clear. In this study we investigated the feasibility of anaerobically co-digesting ground beef hooves in the presence of swine manure or slaughterhouse sludge at 25 °C using eight 42-L Plexiglas lab-scale digesters. Our results showed addition of beef hooves statistically significantly increased the rate of CH_4 production with swine manure, but only increased it slightly with slaughterhouse sludge. After 90-day digestion, 73% of beef hoof material added to the swine manure-inoculated digesters had been converted into CH_4, which was significantly higher than the 45% level achieved in the slaughterhouse sludge inoculated digesters. BODIPY-Fluorescent casein staining detected proteolytic bacteria in all digesters with and without added beef hooves, and their relative abundances corresponded to the rate of methanogenesis of the digesters with the different inocula. Fluorescence in situ hybridization in combination with BODIPY-Fluorescent casein staining identified most proteolytic bacteria as members of genus *Alkaliphilus* in the subfamily *Clostridiaceae* 2 of family *Clostridiaceae*. They thus appear to be the bacteria mainly responsible for digestion of beef hooves.

1. Introduction

Large amounts of organic by-products are produced from slaughterhouse and meat-processing industries because of the high demand for meat resulting from increased global populations and their associated economic growth (Tritt, 1992; Tritt and Schuchardt; 1992; Cho et al., 1995). Canada produced 3.5 billion pounds of beef (1.6 billion kilograms carcass weight) in 2006, which contributed $26 billion to its economy (Canfax, Statistics Canada 2006). Consequently, large quantities of animal by-products (ABPs) including carcasses and products of animal origin rich in proteins and lipids (Edström et al., 2003; Resch et al., 2006), which constitute approx. 25% of total animal weight not intended for human consumption, are generated [Reg. (EC) No. 1069/2009 – replacing the ABP directive 1774/2002]. Traditionally ABPs have been treated by rendering processes and used as animal fodder, thus providing slaughterhouses with additional valuable sources of income (Auvermann et al., 2004). However, outbreak of diseases like bovine spongiform encephalopathy in cattle and Creutzfeld–Jacob in humans has prohibited their utilization as animal fodder. Consequently disposal of ABPs has emerged as a major concern in the meat industry from an increasing awareness of the need for stringent hygiene regulations and tighter process control (Hejnfelt and Angelidaki, 2009).

Anaerobic digestion is an effective method of treating ABPs, at the same time generating energy in the form of CH_4, and the resulting nutrient rich digestion effluents can be used as agricultural fertilizers (Salminen and Rintala, 2002). Successful methanogenesis from anaerobic digestion of poultry by-products like blood, meat and bones (Salminen and Rintala, 2002), rumen digesta and cattle blood (Banks and Wang, 1999), blood and category 3 materials (http://europa.eu/legislation_summaries/food_safety/animal_nutrition/f81001_en.htm) from pigs have been reported (Kirchmayr et al., 2007). However, little information exists for the degradation of keratin-rich materials like feathers, hairs, hooves, horns or toenails. Keratins are categorized as α-keratin (e.g. bovine hooves) and β-keratin (e.g. chicken feathers) consisting of tightly packed protein

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chains in α-helices and β-sheets respectively (Parry and North, 1998). Both are stabilized by high degrees of disulfide and hydrogen bond cross-linking, as well as hydrophobic interactions, which render them insoluble and resistant to biodegradation (Fuchs, 1995).

Few studies have looked at the anaerobic digestion of keratin-rich wastes for biogas production. Hejnfelt and Angelidaki (2009) reported that hair and skin from Danish piggeries supported high CH_4 yields under thermophilic conditions (55 °C) compared to other piggery by-products. We have used untreated chicken feathers (β-keratin wastes) as a model protein for prions to investigate the feasibility of their anaerobic co-digestion with swine manure or slaughterhouse sludge, and found that they could be digested effectively (Xia et al., 2011, 2012a,b), where *Alkaliphilus* spp. were the main feather-hydrolysers. However, similar information for the anaerobic digestion of α-keratin wastes such as hairs, nails, horns and hooves is lacking.

In this study, we have investigated the feasibility of co-digesting ground beef hooves with no pre-treatment, and with addition of swine manure or slaughterhouse sludge at 25 °C, and attempted to identify the keratin-hydrolyzing organisms involving in their degradation using BODIPY fluorescence casein (BODIPY-FluoC) staining combined with fluorescence in situ hybridization (FISH) (Xia et al., 2007).

2. Methods

2.1. Preparation of bovine hoof samples

Fresh bovine hooves (10 kg) were collected from a local slaughterhouse (Colbex, Levinoff, QC, Canada) and transferred to the laboratory within 4 h. The hooves were then washed with deionized water and dried at 45 °C in a Unitherm dryer (Construction CQLTD, England). The weight of each dryer box was recorded daily until a constant weight for each was reached, which took about one week. Subsequently, the hooves were cut manually into smaller pieces of 3–5 cm with a drill and then ground through a 2-mm screen (Thosmas-Wiley, Laboratory Mill). Samples were then ground in a blender (Vita-Mix5200, Vitamix Corporation) and passed through a sieve with a pore-size of 500 μm. Homogenized hooves were divided into aliquots of ≈33 g and placed in nitrogen-free polyester forage bags with a pore size of 50 μm (ANKOM Technology, 2052 O'Neil Road Macedon, NY14502, USA), sealed with plastic tie wraps, and washed in a washing machine (Frigidaire, Martinez, GA, USA) using the delicate cycle setting to remove as much remaining residual material as possible. Washed hoof bags were then dried at 45 °C until their weights were constant (≈31 g each), and were labelled and tied onto a steel stick and placed into the digester (see below).

2.2. Digestion experiments

Two 7-m^3 semi-industrial scale digesters (Massé et al., 1996) were used to stabilize fresh swine manure collected from a commercial pig farm (Sherbrooke, Quebec), and slaughterhouse sludge obtained from a commercial cattle slaughterhouse (Colbex, Levinoff, Quebec). These digesters had been operating at 25 °C for more than 2 years with a mean retention time of 14 d before the inocula were used. Eight 42-L Plexiglas lab-scale digesters described by Massé et al. (2001) were used for hoof incubation experiments. Four 42-L digesters were fed 35-L of the swine manure. Two of these were fed bovine hooves. The other two did not receive beef hooves and served as controls. Four 42-L digesters were fed with 35-L of the slaughterhouse sludge and set up with or without beef hooves as described for the swine manure fed digesters. Addition of the beef hooves was achieved by adding 14 beef hoof bags per digester, representing 28.9%, and 26.7% of the total chemical oxygen demand (COD) loading ratio for the swine manure digesters (SMDs) and slaughterhouse sludge digesters (SSDs) respectively. Digesters were operated in batch mode in a closed room maintained at 25 °C for 90 d. Twelve hoof bags were removed at day 90 from each digester to measure beef keratin degradation. Also, two bags were taken from each digester at different time intervals (22–25 d), sampled for BODIPY-FluoC staining, and returned immediately. Digesters were mixed thoroughly daily and before each sampling for 5 min using a circulation pump.

2.3. Analyses

Total solids (TS), volatile solids (VS), total suspended solids (TSS), volatile suspended solids (VSS), total chemical oxygen demand (COD), soluble COD of manure, and COD, dry matter, organic matter, ash content, protein content of the raw beef hooves were determined according to the standard methods (APHA, 1998) as described previously (Xia et al., 2012a). Biogas composition (CH_4, CO_2, H_2S and H_2), mixed liquor total Kjeldahl nitrogen (TKN), mixed liquor ammonium nitrogen (NH_4^+-N) and soluble volatile fatty acids (VFAs) were analyzed following procedures described by Massé et al. (1996) as detailed previously (Xia et al., 2012a).

2.4. Bacterial sampling for BODIPY-FluoC staining

The sampling and staining of proteolytic microorganisms were carried out according to the procedure described by Xia et al. (2011) with a slight modification. To stain for organisms attached to the hoof particles, fresh hoof samples (ca. 5 g) were collected from a hoof bag after mixing carefully with a sterile glass rod at each sampling point, drained for 3–5 min on a sieve and re-suspended in 50 ml bacteria-free filtrate (Xia et al., 2011). The filtrate was prepared from the mixed liquor of the same digester by centrifugation at 1600*g* for 30 min and filtration through a series of filters (Whatman Nuclepore track-etched membranes) with pore sizes of 3, 1, 0.45, and 0.2 μm, used sequentially. The mixture was then transferred into a thick-walled polyethylene bag (180 × 300 mm) and subjected to vigorous mechanical pummelling for 2 min using a Colworth Stomacher 400 (A.J. Seward & Co., Ltd., London). The mixed liquid was subsequently filtered through 8-layer sterilized cheese clothes and the collected filtrate was centrifuged at 800*g* for 15 min to remove any large particles. Then 200 μl of supernatant, which contained bacterial cells washed from the beef particles, was mixed with the same volume of freshly prepared 2 × Tris-HCl (10 mM, pH 7.8) buffer before addition of 200 μl BODIPY-FluoC working solution. The mixture was incubated in a 10 ml serum bottle wrapped in aluminum paper at 25 °C for 30 min on a rotating platform (220 rpm). Incubated samples were evenly spread on 3-well (10 μl in each well) Teflon-printed slides (Electron Microscopy Sciences, Hatfield, PA, USA) and dried in a dark room before being mounted with CITIfluor (Electron Microscopy Sciences, Hatfield, PA, USA) and examined microscopically.

2.5. Enumeration of microorganisms positively stained with BODIPY-FluoC

Numbers of proteolytic microorganisms were estimated by counting the number of cells positively stained with BODIPY-FluoC as a percentage of the total cell number determined after staining with DAPI (4′, 6-diamidino-2-phenylindoledihydrochloride) in the same microscopic field according to the procedures described previously (Xia et al., 2011). Cells on digital images were counted in ImageJ (Abramoff, et al., 2004). Each image contained ca. 1000–3000 bacterial cells. For each enumeration, at least 60