Bioresource Technology 117 (2012) 124–130

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

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

Distribution of methanogenic potential in fractions of turf grass used as inoculum for the start-up of thermophilic anaerobic digestion

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HIGHLIGHTS

- Thermophilic methanogens are present in various grass turf species.
- Both acetoclastic and hydrogenotrophic methanogens are present.
- Acetoclastic methanogens are mainly present in turf soil.

ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form 19 April 2012
Accepted 20 April 2012
Available online 28 April 2012

Keywords:
Turf
Start-up
Inoculum
Thermophilic anaerobic digestion
Methanogens

ABSTRACT

This study aims to investigate thermophilic methanogens in turf used as an inoculum. Results showed that *Methanoculleus* sp. regarded as hydrogenotrophic and *Methanosarcina* sp. regarded as acetoclastic methanogens were present in turf tested. However, active acetoclastic methanogens were present in turf soil only. The current study showed that thermophilic methanogens were present in various turf grass species: *Stenotaphrum secundatum*, *Cynodon dactylon*, and *Zoysia japonica*. Severe treatments of grass leaves under oxic conditions, including blending, drying and pulverizing did not affect the thermophilic hydrogenotrophic methanogenic activity of the grass. A dried and pulverized grass extract could be generated that can serve as a readily storable methanogenic inoculum for thermophilic anaerobic digestion. The methanogens could also be physically extracted into an aqueous suspension, suitable as an inoculum. The possible contribution of the presence of methanogens on grass plants to global greenhouse emissions is briefly discussed.

1. Introduction

Thermophilic anaerobic digestion provides significant benefits over mesophilic anaerobic digestion, which includes enhanced pathogen destruction (Dugba and Zhang, 1999), greater methane production rate (Griffin et al., 1998; Ahn and Forster, 2000), and faster organic degradation rate (Yilmaz et al., 2008; Khalid et al., 2011). Despite these benefits, application of thermophilic anaerobic digestion has not been widely used due to difficulties in operation and start-up. The start-up of thermophilic anaerobic digestion is the most significant constraint and obtaining a suitable methanogenic inoculum is a key factor for successful start-up.

To overcome the shortage of thermophilic methanogenic seed material, various seed materials have been tested as inocula for the start-up of thermophilic anaerobic digestion. Freshly sampled mesophilic anaerobic sludge is a well-known inoculum used for starting thermophilic anaerobic digestion (Bolzonella et al., 2003; Forster-Carneiro et al., 2008). However, with the transition of temperature from 37 to 55 °C, a significant drop in methanogenic activity is usually observed (Fang and Lau, 1996; Khalid et al., 2011). Some researchers have used endogenous microbes contained within the waste as a sole inoculum for start-up of thermophilic anaerobic digestion (Kim and Speece, 2002; Chachkhiani et al., 2004; Suwannoppadol et al., 2011). Kim and Speece (2002) suggested that waste-activated sludge (WAS) was a proper seed for thermophilic anaerobic digestion. The authors reported that thermophilic methane production of about 0.35 L methane/L reactor/day ($L\,L^{-1}\,d^{-1}$) was obtained after feeding acetate as a carbon source. Moreover, cow manure was used as inoculum for start-up of not only mesophilic (Garcia-Peña et al., 2011) but also thermophilic anaerobic digestion (Chachkhiani et al., 2004). Chachkhiani et al. (2004) succeeded in using cow manure as inoculum to start-up thermophilic anaerobic digestion, leading to a maximum biogas production of $0.2\,L\,L^{-1}d^{-1}$ after 10 days of thermophilic incubation.

According to our previous study (Suwannoppadol et al., 2011), the Organic Fraction of Municipal Solid Waste (OFMSW) contains a suitable inoculum for start-up of thermophilic anaerobic digestion. The source of active thermophilic methanogens was

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http://dx.doi.org/10.1016/j.biortech.2012.04.082

narrowed to down to be part of grass clippings (grass turf) contained in the waste. However, our previous study did not identify species of thermophilic methanogens present in turf due to the limitation of culture-based methods. To enhance insights into the methanogenic communities, the current study identified methanogens present in grass by using molecular-based methods (the polymerase chain reaction (PCR) technique relying on the amplification of 16S rRNA).

Generally, methanogens require an anaerobic or anoxic habitat to survive and flourish. However, turf, in particular the grass leaves, are fully exposed to an aerobic environment. From a biological perspective the presence of oxygen sensitive thermophilic methanogens in a fully aerobic environment was unusual and unexpected. For successful and reliable thermophilic anaerobic digestion of material that contains turf grass it is important to know, which types and which fractions of the grass carry this "free inoculum". From the fact that turf grass can serve as a suitable inoculum for thermophilic anaerobic digestion, it would also be interesting to explore whether the methanogens can be readily stored for example in a dry form and hence enabling the production of concentrated inoculum material for thermophilic anaerobic digestion.

The aims of this study were to:

- Corroborate the findings that turf grass is a key source of thermophilic methanogens in the OFMSW.
- Compare the thermophilic methanogenic activities in different turf grass species.
- Determine the types of methanogens found in/on grass lawn when incubated at different temperature ranges (mesophilic and thermophilic) and when provided with different energy sources.
- Examine the effects of blending, drying and pulverizing of grass leaves on the capacity for methane production
- Identify which part of grass turf (leave or root with surrounding soil) is a source of acetoclastic and hydrogenotrophic methanogens
- Identify methanogens present in turf used as inoculum

2. Methods

2.1. Samples collection and preparation

2.1.1. Inoculum sources

Components of "turf" used in the current study consisted of the turf's grass leaves, roots, and soil (soil attached to the roots). Various combinations of turf grass leaves, and turf soil were tested as a source of inoculum to start-up anaerobic digestion (Table 1). Fresh turf samples were collected from the Murdoch University campus, Perth, Western Australia, or, in the case of Fig. 1, from a local Perth turf supplier (Westland Turf: *Stenotaphrum secundatum*, *Cynodon dactylon*, and *Zoysia japonica*) and used as inocula. Fresh grass leaves were collected at least 2 cm above the soil profile to minimize any contamination by the soil. To prepare turf soil samples (Fig. 4), grass leaves and main grass roots were manually removed. Consequently, all turf soils used contained a limited quantity of fine hair roots. Once sorting was complete, soil samples were used immediately as an inoculum.

2.1.2. Treatment of grass leaves

Three main techniques were used to prepare the grass leaves before testing: blending, drying, and pulverizing. To blend grass leaves, 5 g of leaves and 40 mL of culture medium were blended by a mechanical blender (DēLonghi, model DBL740) for 15 min. To prepare dried grass leaves, 5 g of leaves were oxic-dried either at room temperature or in an oven at 37 °C, for one week. Powdered grass leaves were prepared by drying 5 g of grass leaves at 37 °C for one week and then blending in a mechanical blender (Breville, model BFP50) until the particle size was less than 2 mm. A summary of the treatment methods applied to individual inoculants is shown in Table 1.

2.1.3. Treatment of methanogenic culture

For Section 3.5, methanogenic activities of untreated (not dried) and treated (dried) methanogenic pellets were compared to examine the effects of oxic-desiccation of methanogenic pellets on methane production. To obtain a dried methanogenic pellet, anaerobic culture collected from the incubated grass turf of Section 3.1 was centrifuged (IEC Centra CL3) at 4000 rpm for 10 min. Ten gram of the wet pellet, which contained residues of grass leaves, roots and soil, were dried at 37 °C for 2 days. Next, the dried methanogenic pellet was anaerobically incubated at 55 °C to administer the methanogenic activity test.

2.1.4. Treatment of soil components – methanogen extraction

Mechanical shaking was employed to extract methanogens from the soil. To extract methanogens from soil, 30 g of turf soil and 50 mL of culture medium were mechanically (Stuart flask shaker) shaken (500 oscillations/min) for 15 min. The supernatant (extracted soil solution) and soil after extraction were used immediately as inocula.

2.2. Carbon source, bicarbonate, and culture medium composition

30 mM and 80 mM of acetate concentrations were used as methanogenic carbon source for Sections 3.1–3.5 and 3.6 respectively. 250 mM of sodium bicarbonate ($NaHCO_3$) was used as buffer for Section 3.1–3.4 and 3.6. Culture medium was used for adjusting working volume to 40 and 50 mL for Section 3.1–3.5 and 3.6, respectively. The culture medium contained (per liter): 0.3 g KH_2PO_4, 0.6 g NaCl, 0.1 g $MgCl_2{\cdot}2H_2O$, 0.08 g $CaCl_2{\cdot}2H_2O$, 1.0 g NH_4Cl, 3.5 g $KHCO_3$, 10 mL of vitamin solution, and 5 mL of trace element solution.

Vitamin solution contained (per liter): 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine hydrochloride, 5.0 mg thiamin hydrochloride, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg DL-calcium pantothenate, 0.1 mg vitamin B_{12}, 5.0 mg *p*-aminobenzoate, and 5.0 mg lipoic acid.

Trace element solution contained (per liter): 12.8 g nitrilotriacetic acid, 1.35 g $FeCl_3{\cdot}6H_2O$, 0.1 g $MnCl_4H_2O$, 0.024 g $CoCl_2{\cdot}6H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.1 g $ZnCl_2$, 0.025 g $CuCl_2{\cdot}2H_2O$, 0.01 g H_3BO_3, 0.024 g $Na_2MoO_4{\cdot}4H_2O$, 1.0 g NaCl, 0.12 g $NiCl_2{\cdot}6H_2O$, 4.0 mg $Na_2SeO_3{\cdot}5H_2O$, 4.0 mg $Na_2WO_4{\cdot}2H_2O$.

2.3. Experimental design

All experiments were conducted in 100 mL serum vial (Wheaton) sealed with butyl rubber stoppers and aluminum crimps. Experiments were performed in duplicate and conducted at 55 °C except Section 3.2 where methanogenic activity tests were also performed at 37 °C. To establish anaerobic conditions, the headspaces of all serum vials were flushed with N_2/CO_2 (80%/20%) for 30 s. All samples were incubated in a water bath (Paton, model RW 1812) with shaking (30 oscillations/min). Following the initial set-up, all serum vials were depressurized to atmospheric pressure after the first hour of incubation. The volume of biogas produced was measured using a 50 ml glass syringe (Popper & Sons, Inc.). Experimental conditions for all experiments are summarized in Table 1.