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Suwat Suwannoppadol, Goen Ho, Ralf Cord-Ruwisch

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Distribution of Methanogenic Potential in Fractions of Turf Grass used as Inoculum for the Start-up of Thermophilic Anaerobic Digestion

Suwat Suwannoppadol, Goen Ho*, Ralf Cord-Ruwisch
Faculty of Science & Engineering, Murdoch University, Perth, Western Australia, 6150
*Corresponding author phone: +61 8 9360 2167
E-Mail: g.ho@murdoch.edu.au

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.

Keywords: turf, start-up, inoculum, thermophilic anaerobic digestion, methanogens

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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 ˚C 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-Peňa et al., 2011) but also thermophilc anaerobic digestion

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(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 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:

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- 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

2.0 Materials and 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 Figure 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 (Figure 4), grass leaves

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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 revolutions per minute for 10 min. Ten g 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

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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/minute) 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 $MgCl_2.2H_2O$, 0.08 g $CaCl_2.2H_2O$, 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.

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

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 seconds. All samples were incubated in a water bath (Paton, model RW 1812) with shaking (30 oscillations/minute). 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.

2.4 Analysis

VFA concentration of samples was analyzed by gas chromatography using a Varian Star 3400 equipped with a Varian 8100 auto sampler and a flame ionization detector as described by Walker et al. (2009). The methane concentration in biogas was analyzed by Varian Star 3400 gas chromatograph equipped with a thermal conductivity detector as described by Charles et al. (2009).

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2.6 PCR amplification and clone library analysis

The Archaeal 16S rDNA was amplified using the primer pairs Arch f364 (CCT ACG GGR BGC AGC AGG) and Arch r1386 (GCG GTG TGT GCA AGG AGC) (Skillman et al. 2004). Polymerase chain reaction mixtures (25 µL) consisted of 10 to 20 ng of template DNA, 1x PCR buffer, 2.5 mM of $MgCl_2$, 0.2 mM of mixed dNTPs, 0.5 µM of both primers, 1.0 U of *Taq* DNA polymerase (Promega, Madison, Wi). The following amplification conditions were used in a BioRad MyCycler thermal cycler: initial denaturation at 95 ^{o}C for 7 min, followed by 32 cycles of 95 ^{o}C for 30 s, 58 ^{o}C for 30 s and 72 ^{o}C for 1 min, and final extension at 72 ^{o}C for 7 min. To verify the presence of the amplified gene, 10µL of the PCR products were run on 1% agarose gel electrophoresis, stained with ethidium bromide and visualised under UV light. For the preparation of the cloning reactions, PCR products were purified with the Wizard SV Gel and PCR clean up kit (Promega, Madison, Wi, USA).

Cloning reactions and transformations were performed using the PGEM –T easy Vector System and JM109 competent cells according to the manufacturer instructions (Promega, Madison, Wi). A total of 50 clones from the non-incubated blended grass samples and 53 clones from the incubated blended grass samples were selected for the amplification of the 16S rRNA gene and subsequent digestion with HaeIII restriction enzyme according to manufacturer's instructions (Promega, Madison, Wi). Digested fragments were separated by electrophoresis on 3% agarose gels. Different restriction fragment patterns were assumed to represent different operational taxonomic units (OTUs).

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2.7 DNA sequencing

Clones containing the methanogenic *Archaea* gene which resulted in dissimilar restriction fragments were selected for growth and insert sequencing. Sequencing was performed in an Applied Biosystems 3730 DNA sequencing system (SABC, Murdoch University). The sequences obtained were manually checked and compared to other sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (NCBI: http://blast.ncbi.nlm.nih.gov). The unique methanogen 16S rRNA gene sequences identified in this study have been deposited in the GenBank under accession numbers JF792623 and JF792625-7.

3. Results and Discussion

3.1 The presence of thermophilic methanogens in various species of turf grass

Our previous study (Suwannoppadol et al., 2011) found that thermophilic anaerobic incubation of turf grass, which consisted of a mixture turf grass species, exhibited a significant methanogenic activity within a few days of incubation. On the other hand, there was no methanogenic activity in samples seeded with mixed tree bark, tree leaves, or soil (away from grass lawn). To investigate whether this phenomenon was generic or linked to specific turf grass species, a number of different turf grass species were tested as the sole inoculum of thermophilic methanogens.

Three turf grass species (*Stenotaphrum secundatum*, *Cynodon dactylon*, and *Zoysia japonica*) were anaerobically incubated at 55 ^{o}C. A mixed soil sample (away from lawn) from Murdoch University was used as control. All grass samples started producing

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methane after four days of anaerobic incubation at 55 °C (Figure 1). An average methane production rate of about 0.3 $L*L^{-1}*d^{-1}$ was obtained for all turf samples, suggesting that similar levels of thermophilic methanogens were present in each species of turf grass.

The rate of 0.3 $L*L^{-1}*d^{-1}$ is surprisingly high considering that anaerobic digesters of sewage sludge produce about 0.5 $L*L^{-1}*d^{-1}$ of methane gas. In comparison with the amount of expected methane produced from acetate added (0.74 L/L), the amount of methane produced (~ 7 L/L) from all turf samples were approximately 10 times more than that from acetate added (Figure1). This result indicates that the turf served as an additional carbon source for methane production.

Acetate analysis after 4 weeks of incubation showed that the residual acetate of all grass samples was lower than the initial concentration of acetate added (30 mM) (data not shown) suggesting that acetoclastic methanogesis was present in all grass samples tested.

3.2 Presence of thermophilic and mesophilic methanogens on grass leaves

To test whether the above observed phenomenon of the presence of active methanogens on fresh turf grass is restricted to thermophilic microbes, mesophilic incubations (30 °C) were also carried out and compared to thermophilic ones. Turf grass samples tested above (Fig. 1) included grass leaves, root, and surrounding soil. The current experiment also aims to test whether grass clippings (grass leaves only) support the presence of live methanogens, as it is largely grass leaves that are present in typical street verge collections of MSW in suburban areas such as Perth.

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After four days incubation of grass leaves, methane was produced under both thermophilic and mesophilic conditions (data not shown). In comparison, the initial methane production rate of thermophilic anaerobic digestion was two times higher than that of mesophilic anaerobic digestion. However, the results clearly indicate that grass leaves contain both thermophilic and mesophilic methanogens.

While methanogens are strictly anaerobes and highly sensitive to oxygen entry into laboratory culture vessels, our study shows evidence of the presence of significant numbers of viable methanogens on grass leaves that are exposed to air. This result is in agreement with previous studies reporting that methanogens could survive under oxic stress (Tang et al., 2004; Brioukhanov and Netrusov, 2007; Tholen et al., 2007; Charles et al., 2009). Liu et al. (2008) compared the tolerance of different methanogenic strains to oxic-desiccation in liquid and dried methanogenic cultures, which had been pre-dried by centrifugation to pellets, then dried by a centrifugal evaporator. These methanogenic strains included *Methanobrevibacter arboriphilicus, Methanoculleus olentangyi, Methanosarcina mazei, Methanobacterium formicicum, Methanococcus vannielii* and *Methanoplanus limicola*. The authors reported that most of these methanogenic strains could survive after desiccation under an oxic atmosphere. However, survival rates were not given. In non-quantitative experiments the pre-dried cultures of these methanogenic strains had higher tolerance to oxic-desiccation than the same methanogenic strains in enriched liquid cultures. Moreover, Tholen et al. (2007) even stated that *Methanobrevibacter cuticularis* could be cultivated in the presence of oxygen by

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making use of oxygen as electron acceptor using hydrogen as the electron donor at fractions of up to 10% of the CH_4 production rate.

3.3 Effects of blending grass leaves on methane production activity

Result of section 3.2 showed that grass leaves, which were fully exposed to air, contained both viable mesophilic and thermophilic methanogens. It is not clear how methanogens as obligate anaerobic microbes, can survive in an aerobic environment such as grass leaves. Grass leaves are complicated in structure consisting of many tissues such as mesophyll, veins, and epidermis. It might be possible that methanogens are protected by the grass tissue, perhaps allowing the existence of an oxygen free micro-environment.

To test this hypothesis, grass leaves were blended for 15 min to damage the structure of the leaves. By breaking the physical structure of grass, methanogens in grass leaves would be exposed to oxygen. The methane produced from such blended grass leaves was compared to that of fresh leaves as the positive control.

Results showed that methane was produced from both blended and fresh grass leaves as the sole inoculum (Figure 2A). The lag time and the initial methane production rate of blended grass samples were 4 days and 0.28 $L*L^{-1}*d^{-1}$, respectively, which were comparable to those of the control (Figure 2A). This indicates that structural damage of grass leaves by blending does not affect the rate of spontaneous methane production. This rules out the possibility of the methanogens having been protected from oxygen by the viable grass tissue.

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It is possible that enzymes protecting against oxygen toxicity play a significant role in the survival of methanogens on grass leaves. Oxygen toxicity protecting enzymes such as superoxide dismutase (SOD) and catalase might have provided enzymatic defense against oxygen toxicity and facilitated the survival of methanogens on oxygen-exposed grass leaves. Enzymes protecting against oxygen were also found in various groups of methanogens and are thought to play an important role in microorganism survival after oxygen exposure (Brioukhanov et al., 2000; Shima et al., 1999, 2001). When strict anaerobic microbes are exposed to oxygen, a product of oxygen reduction, the superoxide radical (O_2^-), is produced. Without immediate neutralization of O_2^- by oxygen toxicity protecting enzymes, the superoxide radical, which is a strong oxidant, will damage anaerobic microbes' cells (Brioukhanov et al., 2007). Brioukhanov et al. (2002) investigated the activity of catalase and SOD among *Methanobrevibacter arboriphilus, Methanosarcina barkeri* Fusaro, and *Methanosarcina barkeri* during different growth phases while hydrogen, methanol, and acetate, were used as energy sources. The authors reported that catalase and SOD were found in all these methanogenic species tested. Moreover, the enzymes activities measured in these methanogenic cells were different depending on their energy source and growth stages.

To test for the presence of hydrogenotrophic and acetoclastic methanogesis, hydrogen and acetate were monitored during the anaerobic incubations of grass turf. Within 24 hours of anaerobic incubation of grass leaves, hydrogen was produced and accumulated to levels of approximately 0.5 L/L of both grass samples. Thereafter, the hydrogen level in the biogas decreased (Figure 2B, day 5), coinciding with the onset of methane production suggesting that the initial methane was produced from H_2 utilizing

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methanogens. Residual acetate concentrations of both grass samples were over 135 mM, which was four times higher than the initial acetate concentration added (30 mM). This observation indicates that acetate was initially not degraded into methane possibly due to the absence or low numbers of acetoclastic microbes.

3.4 Effects of drying grass leaves on methane production

Previous experiments confirmed that grass leaves could be effectively used as an inoculum for the start up of methanogenic reactor. Also, methanogens carried by grass leaves were tolerant to aerobic condition. From a practical aspect of using methanogens from grass as an inoculum for thermophilic anaerobic digestion, it would be advantageous if the grass leaves, to be used as the anaerobic inoculum, could be dried as it can minimize transportation and storage expenses. The aim of this experiment was to examine effects of oxic-drying of grass leaves on methane production during their anaerobic incubation.

Drying grass leaves at room temperature and 37 ^{o}C did not affect potential methane production compared to the control (fresh grass leaves). The lag times (4 days) and initial methane production rates (about 0.2 $L_{*}L^{-1}{}_{*}d^{-1}$) of both dried grass samples were similar to those of the control (Figure 3). This result supports previous research reported by Charles et al. (2009). Charles et al. (2009) found that there were viable thermophilic methanogens in fresh MSW (containing grass clippings), which was collected from house verge weekly. Also, the lag time (4 days) and initial methane production rate (about 0.2 $L_{*}L^{-1}{}_{*}d^{-1}$) of the powdered grass sample were comparable to those of the control (fresh grass, Figure 3). The results suggest that powdered dried

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grass could be a good inoculum for thermophilic anaerobic digestion. As in the results with dried and non-dried grass, evidence of acetate degradation was not obtained within a period of two weeks of incubation (data not shown) suggesting that methane is likely produced from hydrogenotrophic methanogenesis.

3.5 Effect of desiccation and oxygen exposure on the survival of methanogens

Figure 3 showed that drying of grass leaves for use as inoculum did not affect the subsequent methane production upon anaerobic incubation of the dried grass. It is possible that the methanogens on the grass have probably been in a desiccated state, hence it is expected that drying of the support material (grass) does not further limit their capacity to produce methane. These results suggest that it could be possible to produce, and dry-store methanogenic cells as back-up large scale inoculum for thermophilic anaerobic digesters. This leads to the question as to whether methanogenic liquid cultures (e.g. from digesters) can survive exposure to oxygen when they are dried.

To address the above question, anaerobic culture taken from incubated grass turf described above was centrifuged to a pellet and exposed to dry air in an oven at 37 ˚C for two days. The main objective was to determine the option of methanogens preservation in the presence of oxygen.

When the dried anaerobic culture was incubated at 55 ˚C, it produced methane gas at a rate of 0.06 $L*L^{-1}*d^{-1}$ (over the first 3 days) which was 3 times lower than the positive control which was not dried (data not shown). This implies that one third of the

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anaerobic culture had survived after desiccation and oxygen exposure. This result is in agreement with previous results reported by Ueki et al. (1997). Ueki et al. (1997) compared the number of methanogens in moist paddy soil before and after air drying for 10 days. The authors reported that about 25% of methanogens in the wet soil remained viable during drying and after storing the dried soil for 4 months.

3.6 Presence and extraction of acetoclastic methanogens in fractions of turf

Results of section 3.3 and 3.4 showed that while methane was produced from the thermophilic incubation of grass leaves acetate was not degraded and accumulated during the two weeks of incubation. This suggests that acetoclastic methanogens are not present in the grass leaves. However, in order to sustain the start-up of anaerobic digestion, the presence of acetoclastic methanogens is required. Based on results of section 3.1 (Figure 1), acetate was only degraded when whole grass turf including leaves and root with surrounding soil were incubated (instead of only grass leaves). This implies that acetoclastic methanogens are mainly present in roots or surrounding soil. To test this hypothesis, the presence of acetoclastic methanogens in the 2 different fractions of grass turf (leaves, roots and soil) was investigated.

Results showed that methane formation occurred in both samples using either grass leaves or roots with surrounding soil as a sole inoculum (Figure 4A). However, acetate was only degraded in the presence of the roots/soil fraction (Figure 4B). This indicates that hydrogenotrophic methanogens are present on the oxygen-exposed leaves whereas acetoclastic methanogens are present in only turf soil. This result leads to a hypothesis that acetoclastic methanogens are more oxygen sensitive than hydrogenotrophic

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methanogens. This result is in agreement with a previous report (Tang et al., 2004). Tang et al. (2004) studied the effects of aeration during the treatment of municipal solid wastes on microbial population dynamics in thermophilic anaerobic reactors. The results showed that the population of *Methanosarcina* in reactors decreased after aeration whereas population of *Methanoculleus* increased based on analysis of the library of 16S rRNA genes clones and the quantitative real-time PCR. Tang et al. (2004) concluded that *Methanoculleus*, a species identified as a hydrogenotrophic methanogen, had a higher tolerance to oxygen exposure as compared to *Methanosarcina*, which is a acetoclasic methanogen.

The reason for the survival of acetoclastic methanogens in soil of grass turf could be because of the presence of oxygen protected microniches in the soil in contrast to the grass leaves, which are fully exposed to oxygen. In the last decade, many researchers have reported the presence of acetoclastic methanogens in soil, such as acidic peatland soil (Steinberg and Regan, 2011) and dried rice paddy soil (Min et al., 1997; Watanabe et al., 2006), which was hypothesized to provide anoxic habitats (Ueki et al., 1997). Wagner et al. (1999) studied the effects of aeration on methane production from soil particles containing methanogens. The authors stated that microscale anoxic areas were formed in soil particles resulting in the survival of methanogens under oxic stress. From a practical aspect of using methanogens from turf soil as an inoculum for thermophilic anaerobic digestion, it would be beneficial if thermophilic methanogens can be extracted for the purpose of preparing a concentrated inoculum. The effect of separating methanogens from the grass by simple mechanical agitation was investigated.

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The methanogens were extracted from the soil by wet extraction as explained in Materials and Methods section. This resulted in 50 ml of extract per 30 g of soil. When compared the methane production by untreated soil with that from the extracted methanogens (data not shown), it can be concluded that methanogens in the turf soil could be extracted by using a mechanical shaker. Under the assumption that the initial methane production rate corresponds to the amount of active methanogens, the rate of methane production from soil extract and the residual soil solution should be lower than that of total untreated soil. However the extract and residual soil after extraction produced similar methane production kinetics to the total untreated soil (data not shown).

3.7 PCR amplification and Clone library analysis

For non-incubated blended grass samples, 29 PCR samples resulted in non specific amplification. Of the remaining 21 fragment patterns from non-incubated blended grass, four distinct sequences were revealed. These phylotypes were closely affiliated within two orders: acetoclastic Methanosarcinales with 36% (18 of 50 clones) of the total clones identified as 97% similar to *Methanosarcina* sp. and hydrogenotrophic Methanomicrobiales with 6% (3 of 50 clones) of the total clones identified as 99% similar to *Methanoculleus* sp. For incubated blended grass samples, 8 PCR samples resulted in non specific amplification. The majority of clones (45 of 53 clones) from the incubated blended grass leaves had very similar fragment patterns. The associated phylotype was placed within the order of hydrogenotrophic Methanomicrobiales with 99% sequence similarity to *Methanoculleus* sp.

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We have demonstrated the presence of methanogens on the unincubated grass and a difference in methanogen composition following incubation. The species identified were most closely related to *Methanosarcina* sp. and *Methanoculleus* sp. In the literature, these two thermophilic methanogenic genera have been found in a thermophilic anaerobic reactor (Sasaki et al., 2006) and cow manure used for start-up of thermophilic anaerobic digestion (Chachkhiani et al. 2003).

This study has confirmed and qualified earlier observations that viable thermophilic methanogenic bacteria can be found on grass leaves and around the roots of turf grass. It has further established that this natural start-up inoculum for thermophilic anaerobic digesters can be "harvested" either as dried grass powder or extracted from the grass as a viable inoculums. How this extract can be suitably preserved as a dry powder remains to be established by further tests.

Aside from the finding that thermophilic methanogens on grass plants serve as an inoculum for anaerobic digestion, the presented findings promise to have significant impact on the understanding of methane release into the environment which is a rising concern due to accelerated global warming.

- Firstly, according to existing knowledge the presence of methanogenic cells on grassland can only be explained by methane production that must have occurred during the growth of the existing cells. Where and under what conditions the methanogens associated to grass have grown could not be answered by this study and warrants further investigation.

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- Secondly, while it is unlikely that the methanogens on grassland produce methane while in contact with air, they will readily produce methane as soon as decaying processes occur such as after cutting of wet grass, exposure of hay to rain, use of cut grass as mulch, composting of green waste including grass.
- Thirdly, it is conceivable that upon digestion by grazing animals, in particular ruminants, the ingested methanogens become active and contribute to elevated methane emissions in the digestion system of grazers.

4. Conclusion

- Thermophilic methanogens are present in various turf grass species.
- Two main groups of methanogens, hydrogenotrophic and acetoclastic methanogens, are present in grass lawn. While only hydrogenotrophic methanogens were present on grass leaves; acetoclastic methanogens were mainly present in the root and surrounding soil.
- Blending and drying grass leaves in an oxic-environment does not affect the methanogens viability
- One third of methanogens cultured from turf survived air drying
- Aqueous extraction with shaking can extract some methanogens from soil into the aqueous phase.

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References

1 Ahn, J.-H., Forster, C.F., 2000. A comparison of mesophilic and thermophilic anaerobic upflow filters. Bioresour. Technol. 73, 201-205.

2 Bolzonella, D., Innocenti, L., Pavan, P., Traverso, P., Cecchi, F., 2003. Semi-dry thermophilic anaerobic digestion of the organic fraction of municipal solid waste: focusing on the start-up phase. Bioresour. Technol. 86, 123-129.

3 Brioukhanov, A.L., Netrusov, A., Sordel, M., Thauer, R.K., Shima., S., 2000. Protection of *Methanosarcina barkeri* against oxidative stress: identification and characterization of an iron superoxide dismutase. Arch. Microbiol. 174, 213-216.

4 Brioukhanov, A.L., Thauer, R.K., Netrusov, A.I., 2002. Catalase and superoxide dismutase in the cells of strictly anaerobic microorganisms. Microbiology 71(3), 281-285.

5 Brioukhanov, A.L., Netrusov, A.I., 2007. Aerotolerance of strictly anaerobic microorganisms and factors of defense against oxidative stress: A review. Appl. Biochem. Microbiol. 43(6), 567-582.

6 Chachkhiani, M., Dabert, P., Abzianidze, T., Partskhaladze, G., Tsiklauri, L., Dudauri, T., Godon, J.J., 2004. 16S rDNA characterisation of bacterial and archaeal communities during start-up of anaerobic thermophilic digestion of cattle manure. Bioresour. Technol. 93, 227-232.

7 Charles, W., Walker, L., Cord-Ruwisch, R., 2009. Effect of pre-aeration and inoculums on the start-up of batch themophilic anaerobic digestion of municipal solid waste. Bioresour. Technol. 100, 2329-2335.

8 Dugba, P.N., Zhang, R., 1999. Treatment of dairy wastewater with two-stage anaerobic sequencing batch reactor systems-thermophilic versus mesophilic operations. Bioresour. Technol. 68, 225-233.

9 Fang, H.H.P., Lau, I.W.C., 1996. Startup of thermophilic (55^{o}C) UASB reactors using different mesophilic seed sludges. Water Sci. Technol. 34 (5-6), 445-452.

10 Forster-Carneiro, T., Pérez, M., Romero, L.I., 2008. Anaerobic digestion of municipal solid wastes: Dry thermophilic performance. Bioresour. Technol. 99, 8180-8184.

11 Garcia-Peña, E.I., Parameswaran, P., Kang, D.W., Canul-Chan, M., Krajmalnik-Brown, R., 2011. Anaerobic digestion and co-digestion process of vegetable and fruit residues: Process and microbial ecology. Bioresour. Technol. 102, 9447-9455.

12 Griffin, M.E., McMahon, K.D., Mackie, R.I., Raskin, L., 1998. Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. Biotechnol Bioeng. 57 (3), 342-355.

13 Khalid, A., Arshad, M., Anjum, M., Mahmood, T., Dawson, L., The anaerobic digestion of solid organic waste. Waste Manage. 31, 1737-1744.

14 Kim, M., Speece, R.E., 2002. Aerobic waste activated sludge (WAS) for start-up seed of mesophilic and thermophilic anaerobic digestion. Water Res. 36, 3860-3866.

15 Liu, C.T., Miyaki, T., Aono, T., Oyaizu, H., 2008. Evaluation of methanogenic strains and their ability to endure aeration and water stress. Curr. Microbiol. 56, 214-218.

16 Min. H., Zhao, Y.H., Chen, M.C., Zhao, Y., 1997. Methanogens in paddy rice soil. Nutr. Cycl. in Agroecosys. 49, 163-169.

ACCEPTED MANUSCRIPT

17 Sasaki, K., Haruta, Shin., Ueno, Y., Ishii, M., Igarashi, Y., 2006. Archaeal population on supporting material in methanogenic packed-bed reactor. J Biosci Bioeng. 102 (3), 244-246.

18 Shima, S., Netrusov, A., Sordel, M., Wicke, M., Hartmann, G.C., Thauer, R. K., 1999. Purification, characterization, and primary structure of a monofunctional catalase from *Methanosarcina barkeri*. Arch Microbiol. 171, 317-323.

19 Shima, S., Sordel-Klippert, M., Brioukhanov, A., Netrusov, A., Linder, D., Thauer, R.K., 2001. Characterization of a Heme-Dependent Catalase from *Methanobrevibacter arboriphilus*. Appl. Environ. Microbiol. 67 (7), 3041-3045.

20 Skillman, L.C., Evans, P.N., Naylor, G.E., Morvan, B., Jarvis, G.N., Joblin, K.N., 2004. 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. Anaerobe. 10, 277-285.

21 Steinberg, L.M., Regan, J.M., 2011. Response of lab-scale methanogenic reactors inoculated from different sources to organic loading rate shocks. Bioresour. Technol. 102, 8790-8798.

22 Suwannoppadol, S., Ho, G., Cord-Ruwisch R., 2011. Rapid start-up of thermophilic anaerobic digestion with the turf fraction of MSW as inoculum. Bioresour. Technol. 102, 7762-7767.

23 Tang, Y., Shigematsu., T., Ikbal., Morimura, S., Kida, K., 2004. The effects of micro-aeration on the phylogenetic diversity of microorganisms in a thermophilic anaerobic municipal solid-waste digester. Water Res. 38, 2537-2550.

24 Tholen, A., Pester, M., Brune, A., 2007. Simultaneous methanogenesis and oxygen reduction by *Methanobrevibacvivacter cuticularis* at low oxygen fluxes. FEMS Microbiol Ecol. 62, 303-312.

25 Ueki, A., Ono, K., Tsuchiya, A., Ueki, K., 1997. Survival of methanogens in air-dried paddy field soil and their heat tolerance. Water Sci. Technol. 36 (6-7), 517-522.

26 Wagner, D., Pfeiffer, E.-M., Bock, E., 1999. Methane production in aerated marshland and model soils: effects of microflora and soil texture. Soil Biol. Biochem. 31, 999-1006.

27 Walker, L., Charles, W., Cord-Ruwisch R., 2009. Comparison of static, in-vessel composting of MSW with thermophilic anaerobic digestion and combinations of the two processes. Bioresour. Technol. 100, 3799-3807.

28 Watanabe, T., Kimura, M., Asakawa, S., 2006. Community structure of methanogenic archaea in paddy field soil under double cropping (rice-wheat). Soil Biol. Biochem. 38, 1264-1274.

29 Yilmaz, T., Yuceer, A., Basibuyuk, M., 2008. A comparison of the performance of mesophilic and thermophilic anaerobic filters treating papermill wastewater. Bioresour. Technol. 99, 156-163.

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Summary of Table and figure captions

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Table 1: Summary of inoculum types, their treatment methods, and initial bicarbonate concentrations used in experiments.

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Figure 1: Comparative methane production over four weeks of incubation in duplicate serum vials of different grass turf species at concentrations of 250 g/L: *Cynodon dactylon* (sample 1 (—▲–) and sample 2 (--▲--)), *Zoysia japonica* (sample 1 (–□—) and sample 2 (--□--)), *Stenotaphrum secundatum* (sample 1 (–◊—) and sample 2 (--◊--)) and turf (sample 1 (–●—) and sample 2 (--●--)) and mixed soil (away from grass lawn) (sample 1 (–X—) and sample 2 (--X--)) from Murdoch University.

Figure 2: Comparative methane production (A) and percent methane and hydrogen gas in the biogas (B) of batch thermophilic anaerobic digestion utilizing 125 g/L of fresh of grass leaves in duplicate serum vials. Legend (A): Untreated leaves (sample 1 (–○—) and sample 2 (--○--)) and blended leaves (sample 1 (–■—) and sample 2 (--■--)). Legend (B): closed symbols: blended leaves, open symbols: untreated leaves. Percent CH_4 ■○, Percent H_2 ▲△ in the biogas.

Figure 3: Comparative methane production (A) during two weeks of incubation in duplicate serum vials at 55 ^{o}C of 125 g/L of various forms of grass leaves as inocula: fresh grass leaves (sample 1 (–●—) and sample 2 (--●--)), grass leaves dried at room temperature (sample 1 (–■—) and sample 2 (--■--)) and 37 ˚C (sample 1 (–△—) and sample 2 (--△--)), and powdered grass (sample 1 (–◊—) and sample 2 (--◊--)).

Figure 4: Comparative methane production (A) and acetate profiles (B) of batch thermophilic anaerobic digestion in duplicate serum vials using 100 g/L of grass leaves (sample 1 (–●—) and sample 2 (--●--)) or 300 g/L of root with surrounding soil of turf (sample 1 (–○—) and sample 2 (--○--)) as a sole inoculum.

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Table 1: Summary of inoculum types, their treatment methods, and initial bicarbonate concentrations used in experiments.

Experiment	Types of inocula	Treatment method	Bicarbonate concentration added (mM)	Experimental duration (Week)	Final working volume (mL)
3.1	10 g of various turf grass species	None	250	4	40
3.2	5 g of grass leaves	None	250	2	40
3.3	5 g of grass leaves	None	250	2	40
	5 g of blended grass leaves	Mechanical blending	250	2	40
3.4	5 g of grass leaves	None	250	2	40
	Dried grass leaves (from 5g of fresh grass leaves)	Air dried at room temperature or in an oven at 37 ˚C for a week	250	2	40
	Powdered grass leaves (from 5g of fresh grass leaves)	Air dried in an oven at 37 ˚C and mechanical blending	250	2	40
3.5	10 g pellet of methanogenic culture seeded with turf	None	0	1	40
	10 g dried pellet of methanogenic culture seeded with turf	Air dried in an oven at 37 ˚C for 2 days	0	1	40
3.6	5 g of grass leaves	None	250	4	50
	15 g of turf soil	None	0	4	50
	30 g of turf soil	None	0	1	50
	50 ml of soil solution	Mechanical shaking	0	1	50
	30 g of turf soil after extraction	Mechanical shaking	0	1	50

The initial pH of all experiments was between 7.8 and 8.4.

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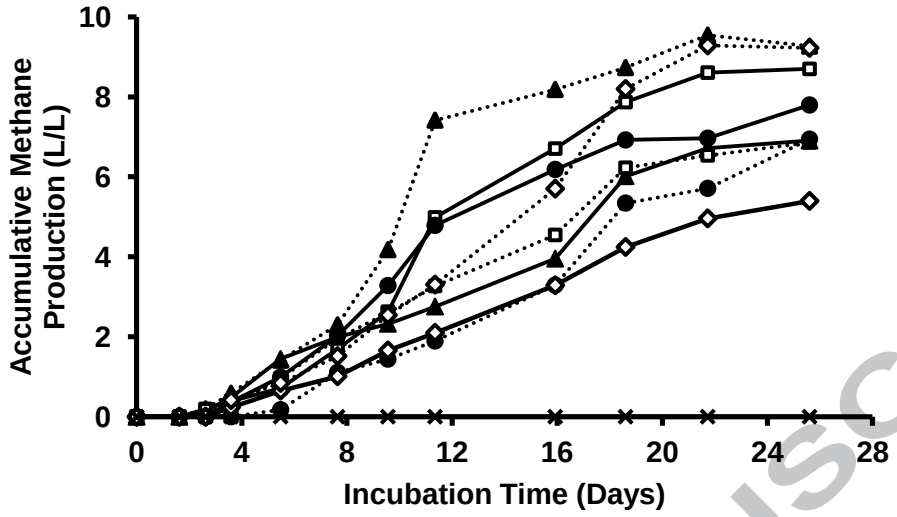

Figure 1

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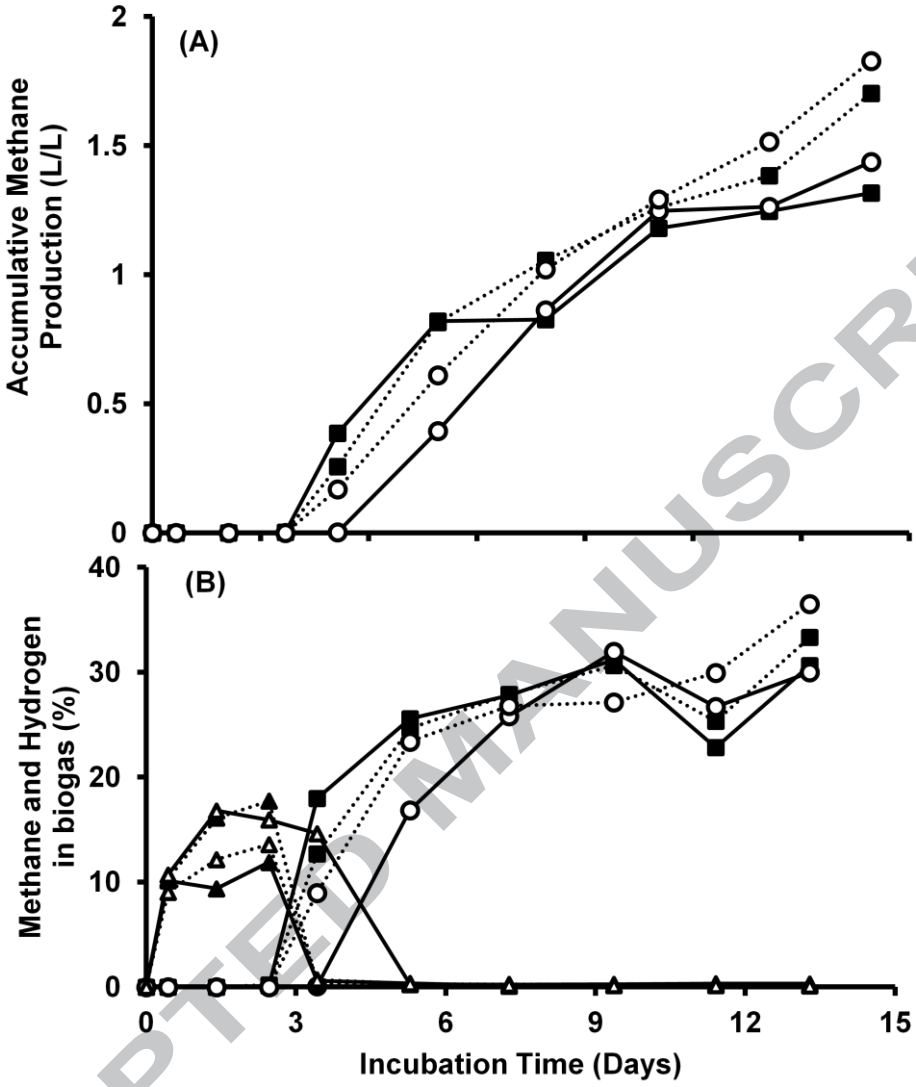

Figure 2

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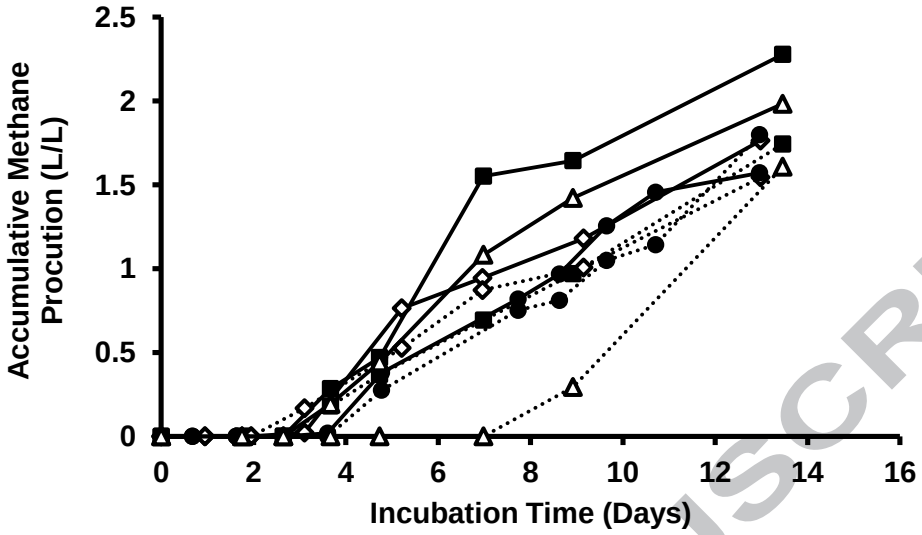

Figure 3

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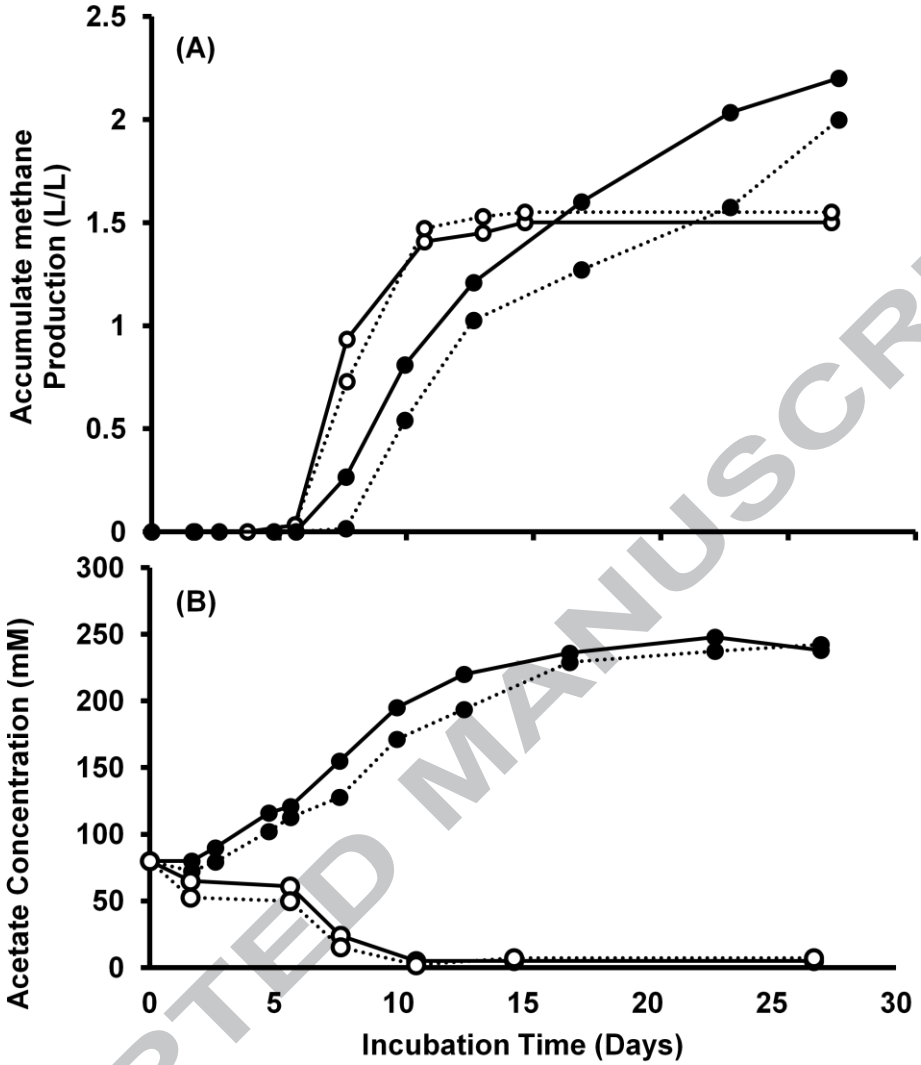

Figure 4

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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.

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