Preservation of Methanogenic Cultures to Enhance Anaerobic Digestion

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PRESERVATION OF METHANOGENIC CULTURES TO ENHANCE ANAEROBIC DIGESTION

by

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ABSTRACT

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The use of anaerobic biotechnology is increasing as a sustainable process to treat various organic wastes. Methanogens convert organic COD into CH_4 and play the key role to drive thermodynamically unfavorable biochemical fermentation reactions and keep the digestion process steady and efficient. Progressive understanding of anaerobic microbiology with digester functionality may help to develop efficient, customized methanogenic cultures to enhance anaerobic bioprocesses. Preservation of methanogenic cultures via drying would be a cost-effective option for research and practical applications. However, preservation of methanogenic cultures is challenging due to methanogen sensitivity to O_2 toxicity and drying, and very limited work is reported on their preservation. The work described herein involves preservation and subsequent storage of various methanogenic cultures in oxic conditions as well as applications to improve performance of anaerobic digesters and standardize laboratory testing.

Five methanogenic cultures were customized under different growth conditions. The cultures were preserved using freeze- and heat-drying, and subsequently stored for short and long periods in the presence of air. Their activity was then assayed by measuring specific methanogenic activity. The influences of growth conditions and protective agent addition were investigated to improve methanogenic activity after preservation. Clone library and qPCR techniques were used to identify and quantify methanogenic communities before and after drying. The usefulness of preserved cultures was examined to bioaugment transiently upset anaerobic digesters and as seed inocula for a standard laboratory test, the biochemical methane potential (BMP) assay. The effect of bioaugmentation was correlated with methanogenic community structure using the DGGE molecular fingerprinting technique.

All customized methanogenic cultures were significantly active even after handling, drying and subsequent storage in the presence of air, suggesting methanogenic culture preservation and storage in air is feasible. Freeze-dried cultures maintained higher methanogenic activity than heat-dried cultures. The culture developed in the presence of limited O_2 exhibited higher methanogenic activity than cultures developed in strict anaerobic conditions regardless of the drying method employed. Glucose as a protective agent resulted in higher methanogenic activity, more so in freeze drying than heat drying. Some methanogenic community members were found to be more tolerant to drying stress than others. Dried methanogenic cultures were found to be viable options to use as a bioaugment to improve treatment efficiency of anaerobic digesters after toxic upset and for the BMP assay.

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Chapter 1 Introduction

Anaerobic biotechnology is a multi-stage process in which complex organic compounds are converted to CH_4. Methanogenesis requires the presence of a specific microbial community composed of different trophic groups in syntrophic relationships (Speece, 2008). The different communities present in various bioreactors are complex and relatively difficult to define. Therefore, the typical, current engineering approach is to pump waste into a digester and rely on the microorganisms that predominate. However, molecular tools are now being used to identify the organisms involved and understand the link between microbial community structure and digester function or activity (Angenent et al., 2002; Tale et al., 2010; Morris, 2011). It is possible that, in the near future, methanogenic microbial communities will be customized for different anaerobic biotechnology applications.

In general, methanogens are slow-growing organisms and require significant time to reproduce as compared to fermentative bacteria. Therefore, it would be beneficial if various defined methanogenic cultures could be preserved for research and addition to anaerobic bioprocesses. Preserved methanogenic cultures could be developed for the following: (1) future research or easy shipment to other laboratories, (2) dissemination and use for standardized tests, such as biochemical methane potential and anaerobic toxicity assays, (3) seeding or re-seeding of laboratory and full-scale reactors, and (4) bioaugmentation of full-scale digesters to increase biogas production and process stability.

The use of wet cultures may be incompatible with some commercial needs due to handling of high volumes, distribution cost and loss of culture activity during storage. To

develop a commercial market and world-wide distribution for beneficial microorganisms, they must be produced in a form that can be economically transported and stored without significant viability loss. Drying reduces the mass and facilitates less expensive shipping and handling and can provide a relatively stable product (Aguilera and Karel, 1997).

1.1   Preservation of microorganisms by drying

Preservation of microorganisms by drying has been a preferred method for decades (Morton and Pulaski, 1938). In bio-industries, mainly spray drying, fluidized bed drying, and air drying are employed on a commercial scale due to their continuous or semi-continuous operation at higher temperature and atmospheric pressure, whereas freeze drying and liquid drying are preferred on small to moderate scale due to their batch operation at lower temperature and negative pressure (Peighambardoust et al., 2011; Santivarangkna et al., 2007; Claussen et al., 2007; Morgan et al. 2006; Ratti, 2001).

One of the advantages of high temperature air drying at a constant pressure is the ability of air to create a high water vapor pressure gradient in the system (the main driving force in drying) and withdraw more moisture from the product in a given time (Claussen et al., 2007). Therefore, high-temperature drying reduces drying time to attain desired low residual moisture content (RMC) in the final product (Santivarangkna et al., 2007). This helps to provide a more stable product during storage (Santivarangkna et al., 2007). For instance, spray drying is a high-temperature process typically performed at an outlet product temperature of 80 to 105 ^{0}C (Desmond et al., 2002; Gardiner et al., 2000). High-temperature spray drying provides faster drying and makes a high-throughput, economical process to preserve some probiotic bacteria than other drying methods (Morgan et al., 2006; Santivarangkna et al., 2007; Peighambardoust et al., 2011).

However, use of high temperature drying techniques for long-term preservation of various microorganisms is very limited due to high cell viability loss of heat-sensitive bacteria at higher process temperatures.

On the other hand, freeze drying is performed at ultra-low temperature and pressure (King et al., 1998; Porubcan, 1991; Simione and Brown, 1991) which avoids denaturing of cells and provides less damage to delicate heat-sensitive microorganisms (Staab and Ely, 1987; Malik, 1990; King et al., 1998; Morgan et al., 2006; Santivarangkna et al., 2007). However, low operating temperature and pressure of the freeze drying method involves long drying time as well as high energy and capital costs (Claussen et al., 2007; Santivarangkna et al., 2007; Peighambardoust et al., 2011). Although freeze drying is a conventional technique for preserving various microorganisms, some microbes, including some anaerobes, are sensitive to the process due to the subzero freezing followed by drying (Malik, 1990a; Malik, 1992; Malik, 1998; Malik, 1999).

Liquid-drying is more helpful to preserve microorganisms which are sensitive to freezing or can require less drying time than freeze-drying (Malik 1990a; Malik 1992; Iino and Suzuki, 2006). Most of the work with liquid-drying is limited to laboratory study only.

All drying technologies have advantages and disadvantages in terms of their economic feasibility and preservation of cell viability. No drying technique is without viable cell loss during preservation and all have their own limitations with respect to preservation of different microorganisms. Furthermore, there is no defined protocol available for generic drying of microorganisms. Morgan et al. (2006) wrote that

"preservation of microorganisms appears to still be a science based on empirical testing rather than facts and tested theories. The drying methodologies could be different for different species even different for strains of the same species". Survivability of microorganisms after drying and during subsequent storage depends on various factors described below.

1.1.1   Type of microorganisms

Different species within the same genus or different strains of the same species may vary in their preservation capabilities even under similar freezing, drying and subsequent storage conditions. Owen et al. (1989) showed that freeze-drying of different strains of *Campylobacter pylori* resulted in different cell viability. The type strain (NCTC 11637) survived better than strain NCTC 11638, and the poorest recovery was found for strain NE 26695. Miyamoto-Shinohara et al. (2000) reported survival rates of 10, 50 and 80 % for yeast, gram-negative and gram-positive bacteria after freeze-drying, respectively. The different survival rate of gram-positive bacteria may be due to the difference in the cell wall membrane as compared to gram-negative bacteria. Others have reported in literature reviews that differences in cell survivability could be due to differences in morphological and physiological characteristics (Carvalho et al., 2004; Morgan et al., 2006; Santivarangkna et al., 2007). Some studies correlated cells size with survival after freezing, drying and storage. Small spherical streptococci cells survival was higher after freeze-drying than long-rod *Lactobacilli* cells (Santivarangkna et al., 2007). A lower surface area to volume ratio may result in less damage to the cell membrane during freezing, drying and storage. However, Palmfeldt and Hahn-Hagerdal (2000) found differences in cell viability of *Lactobacillus reuteri* after drying at the stationary growth phase, and the shape of the cells did not vary. Author also suggested that besides

morphological variation, genetic differences may result in production of different stress-induced proteins and endurance in different cells.

1.1.2 Cell concentration

To ensure sufficient viable cells remain after drying, storage and reconstitution, initial cell concentration greater than 10^7 cells/mL was considered to be required for most preservation methods (Malik, 1990; King et al., 1998; Miyamoto-Shinohara et al., 2000; Morgan et al., 2006). Miyamoto-Shinohara et al. (2000) reported that viable cell concentrations more than 10^4 cells/mL were sufficient for cell revival.

1.1.3 Growth phase

Cell growth is categorized in four phases: lag, log, stationary, and death phases in a batch culture. Growth phase influences survivability of microorganisms during drying. The optimal growth phase for higher drying survival was found to be largely dependent on the type of organism (Morgan et al., 2006; Santivarangkna et al., 2007). Corcoran et al. (2004) demonstrated higher survival of spray-dried *Lactobacillus rhamnosus* (31to 50%) during the stationary phase compared to cells from the log (14%) and lag phases (2%). Higher survival of dried cells from the stationary phase was anticipated due to the cell stress response exhibited during starvation that ostensibly improved the cell survival during the stressful drying conditions. In contrast, Boumahdi et al. (1999) observed higher survival for cells from the lag phase compared to log and stationary phases for air-dried *Sinorhizobium* and *Bradyrhizobium* strains.

1.1.4 Growth media

Preservation studies have shown that the growth media can have a significant effect on survivability of microorganisms after drying and subsequent storage (Carvalho et al., 2004; Morgan et al., 2006; Santivarangkna et al., 2007). Carvalho et al. (2004) discussed

that growth media with sugar developed cells with different morphological and physiological qualities which reflect distinct tolerances to stressful conditions like drying and subsequent storage. Carvalho et al. (2003) showed the higher survival rate of freeze-dried *Entercoccus durans* when grown in de Man, Rogosa and Sharpe (MRS) ager medium compared to M17 agar medium, whereas results were different for freeze-dried *Entercoccus faecalis* which showed higher survival when grown in M17 compared to MRS ager media. Streeter (2003) discussed the effectiveness of trehalose in a growth medium for higher protection of *Bradyrhizobium japonicum* against drying stress compared to using it as a protective agent added after growth, but just before drying. Higher trehalose concentrations in the growth medium were related to accumulation of trehalose in the cell cytoplasm which stabilized the cell cytoplasmic membrane during drying. In contrast, Santivarangkna et al. (2007) reported that addition of trehalose to growth medium did not improve the viability of *Streptococcus thermophilus* after spray drying; cells grown in lactose showed 44% higher viability than cells grown in trehalose. The study conducted by Carvalho et al. (2004) demonstrated the effectiveness of different growth additives to improve survivability of freeze-dried *Lactobacillus bulgaricus* during storage. The cell viability was higher in mannose growth medium than fructose, lactose or glucose. A similar effect was not seen when the cells were tested for thermotolerence. Cells grown in lactose growth medium showed the highest resistance to heat stress.

Morgan et al. (2006) discussed the use of compatible solutes such as polyols, amino acids and amino derivatives as additives for growth media to improve the survivability of cell during drying. Addition of compatible solutes can stabilize the cells during drying by counterbalancing the intra- and -extra cellular osmotic pressure.

1.1.5   Stress-induced growth conditions

Microorganisms have an ability to defend against various chemical and physical stresses and sustain viability in harsh conditions. Carvalho et al. (2004) divided the microbial defensive systems into two classes. In the first class, defensive systems protect the cells from the sudden evolved stress until the stress is removed, whereas in the second class, defensive systems assist the cells to adapt to continuous environmental stresses which may not have been previously encountered by the cells. Induction of stress in cell growth conditions can lead to the growth of cells with different morphological and physiological traits which can enhance the resistance and/or resilience ability to tolerate stressful conditions such as heat or osmotic stresses during drying. Santivarangkna et al. (2007) discussed that sub-lethal heat shock prior to drying improved the survivability of *Lactobacillus delbrueckii spp. bulgaricus* and *Lactobacillus paracasei* after spray drying with an outlet temperature of 100 to 105 ^{0}C. Palmfeldt and Hahn-Hagerdal (2000) demonstrated higher viability of *Lactobacillus reuteri,* showing 90% cell recovery, by lowering the pH from 6 to 5 which induced a greater protection to cells to handle freeze-drying stress compared to 65% recovery after freeze-drying at a pH of 6. Maus and Ingham (2003) reported higher survivability of a probiotic strain of *Bifidobacterium* by starving the cells or reducing the pH of growth media from 6.2 to 5.2, suggesting that cell growth in stressful conditions induces genes that encode for stress-responsive proteins that protect during drying (Morgan et al., 2006).

1.1.6   Drying methodology

Drying has the conflicting outcomes of cell inactivation due to low RMC that imposes a severe stress during drying, and lower RMC that provides higher cell stability during storage. For example, drying of cells below an RMC of 20 to 25% (dry mass weight)

exerts severe stress which can significantly damage the cellular structure and proteins (Santivarangkna et al., 2008a), whereas an RMC of 10% (dry mass weight) was reported to be the optimal value for long-term stability of cells during storage (Aguilera and Karel, 1997).

Authors discussed (Wolfe, 1987; Santivarngkna et al., 2008a) low RMC can significantly damage the cellular structure and various important biomolecules such as protein, RNA and DNA due to osmotic imbalance between intra- and extra- cellular environments. However, the cellular lipid membrane is typically considered to be the primary target of drying-induced damage. Drying removes the hydrogen bonded water molecules attached to the phospholipid bilayer of the cell membrane. Increases in the removal of hydrogen-bonded water causes a higher compressive stress on the cell phospholipids bilayer due to the van der Waals attractive forces pulling the acyl chain together; as a result, the cell membrane undergoes a phase transition from liquid to gel phase. This leads to significant leakage of the cell membrane. The larger effect of compressive stress in the cell membrane may occur when RMC proceeds below 25% mass dry weight and it can be detected when the moisture content is less than 20% mass dry weight in the form of a gradual increase in transient membrane temperature (T_m) (Bryant et al., 2001; Santivarngkna et al., 2008a). The T_m is the transition temperature of cell membrane from liquid to solid or vice versa.

1.1.6.1 Freeze drying

Freeze-drying is performed in two steps: freezing followed by drying. In freezing, water associated with the sample is converted into ice and in drying, water is removed from ice

(solid) to vapor (gas) at low temperature and low pressure by the sublimation process. This process is also called lyophilization (Tan et al., 2007).

Freeze drying is a widely-used technique for preserving and storing various heat-sensitive microbial cultures in the laboratory as well as in large-scale bio-industries. The American Type Culture Collection (ATCC) uses freeze drying for preservation of various bacteria, archaea, yeast, algae, protozoa, and fungi (Alexander et al., 1980; Simione and Brown, 1991).

King et al. (1998) successfully preserved the viability of *Lactobacillus acidphilus* using freeze-drying. Miyamoto-Shinohara et al. (2000) investigated the survival rates of 10 species of yeast as well as gram-positive and gram-negative bacteria immediately after freeze-drying. Overall survival rates for yeast, gram-negative and gram-positive bacteria after freeze-drying were approximately 10, 50, and 80 %, respectively. Malik (1988) described successful preservation of more than 160 strains representing 36 species of nitrogen-fixing bacteria, 11 species of chemolithoautotrophic bacteria and five species of *Aquaspirillum.* All tested strains proved viable and showed 10 to 100% survival after freeze-drying in protective media. In such lyophilized cultures, no loss in plasmids or other desirable characteristics was observed. Teather (1982) demonstrated more than 60% survival of 12 obligately anaerobic rumen bacteria strains after freeze-drying and 2 years of subsequent storage at 4 ^{0}C under vacuum. Malik (1990) showed that anaerobic bacteria which are sensitive to light and oxygen can be preserved using freeze-drying with addition of activated charcoal in cell suspension as a protective media prior to freezing. Fletcher and Young (1998) preserved plant pathogenic bacteria viability by one to two orders of magnitude less than its original population after freeze-drying. McGrath

et al. (1978) concluded that freeze-drying is an effective method for preserving some

algal strains. Suzaki et al. (1978) used freeze-drying to preserve protozoan cells. Tan et

al. (2007) discussed that majority of 50,000 fungal strains were successfully freeze-dried

at the Central Bureau voor Schimmelcultures (CBS) culture collections.

Freezing

In freezing, water associated with the sample is frozen into ice either by slow or rapid

freezing. In slow freezing, the sample is cooled down slowly from its ambient

temperature to its eutectic temperature at a controlled rate of between 1 to 10 ^{0}C/min

(Malik, 1990; Simione and Brown, 1991; Porubcan, 1991). Eutectic temperature is that

temperature at which all liquid in a sample is solidified in ice crystals. In general, a

freezing sample contains a mixture of one solvent and more than one solute; the solute

concentration changes as the water freezes which changes the eutectic temperature. The

water and some solutes crystalize sooner than other crystallizable dissolved solutes. The

solutes go to a glassy state first and then form crystals at a specific eutectic temperature

which lowers the freezing temperature. For effective freeze-drying, the sample must be

cooled until all of the eutectic mixtures are frozen and slow molecular motion of the non-

crystalline portion of the mixture (Simione and Brown, 1991) which is typically done by

cooling the cell suspension to at least -30 to -45 ^{0}C (Malik, 1990; Tan et al., 2007).

Teather (1982) discussed the range of -40 to -80 ^{0}C for freezing or freeze-drying for

adequate preservation of anaerobic rumen bacteria.

In rapid freezing, the sample is frozen by directly immersing the sample in ultra-

low temperature liquid medium. Several ultra-low temperature liquid media are used for

rapid freezing (Table 1.1) , but the most commonly used liquid medium for rapid freezing

is liquid nitrogen (Staab and Ely 1987; Miyamoto-Shinohara et al., 2000; Tan et al.,

2007). A number of microorganisms are routinely stored in liquid nitrogen in type culture

collections (Dahmen et al., 1983). Staab and Ely (1987) employed a rapid-freezing

method using liquid nitrogen to freeze sensitive anaerobic bacteria. In this method, the

cultures were swirled in a flask immersed in liquid nitrogen and frozen as a thin shell on

the inner surface of the flask. The cell viability was found to be greater than 50% for all

freeze-dried cultures.

Drying

The freeze-drying cycle proceeds in two stages: primary drying and secondary drying. In

primary drying, the frozen water is removed by sublimation of ice crystals to water vapor.

The end of the primary drying is either detected by using some instrument or by

empirical testing. Tan et al. (2007) discussed that the end of primary drying is detected by

a pressure rise when the valve between the condenser and the drying chamber is closed. If

the pressure increases after valve closure, then not all the frozen water is evaporated. If

the pressure rise test cannot be performed, then primary drying should last approximate

16 hour when the thickness of the layer of material to be dried is 1 to 2 mm.

The major fraction of water is removed during primary drying, but some bound

water remains in the dried product. Bound water is trapped within the solid matrix and

can take a long time to remove. The bound water is removed during secondary drying at

low pressure and low condenser temperature. Secondary drying reduces the RMC and

increases the stability of final products (Simione and Brown, 1991). Ideally, the end-point

of secondary drying is determined by analysis of the RMC within the freeze-dried

products (Morgan et al., 2006). Often, secondary drying is performed for the same length

of time as primary drying if the resulting RMC is found to be acceptable (Porubcan, 1991).

Successful preservation of various microorganisms using freeze drying at different condenser temperatures, vacuum pressures and drying times was discussed by many authors and is presented in Table 1.1.

Table 1.1. Freeze drying parameters

Type of culture	Species/Genera	Freezing		Drying			Reference
		Slow/controlled freezing	Rapid/deep freezing	Condenser Temperature (^{0}C)	Pressure (Pa)	Drying time	
Anaerobic bacteria	---	---	Liquid nitrogen (-196 ^{0}C)	-45 ^{0}C	13.3 Pa	5-6h	Staab and Ely, 1987
Anaerobic phototrophic bacteria	---	-40 ^{0}C (1-2 ^{0}C/min)	---	---	670 Pa	12-16h	Malik, 1990
American type culture collection protocol (ATCC) for freeze drying	---	-40 ^{0}C to -90 ^{0}C (1-10 ^{0}C/min)	Liquid nitrogen (-196 ^{0}C)	-60 to -70 ^{0}C	400 Pa	---	Simione and Brown, 1991
--	---	---	Ethylene glycol-dry ice slush (-78 ^{0}C)	-78 ^{0}C	350 to 400Pa	Over night	Porubcan, 1991
Aerobic	*Lactobacillus acidophilus*	-40 ^{0}C overnight	---	-60 ^{0}C	<13000Pa		King et al., 1998
Anaerobic	---	-20 ^{0}C	---	---	---	8 h	Castro et al., 2002
Aerobic	*Lactobacillus bulgaricus*	-80 ^{0}C	---	---	670 Pa	---	Teixeria et al., 1995
Aerobic	*Escherichia coli, Bacillus thuringiensis*	---	Dry-ice-methanol mixture	-80 ^{0}C	13 to 130 Pa	---	Leslie et al., 1995
Aerobic	*Lactococcus, Streptococcus*	---	Frozen in dry ice and stored at (-20 ^{0}C) overnight	-53 ^{0}C	90000 Pa	24h	To and Etzel, 1997
Aerobic	*Bacillus, Corynebacterium, Streptococcus, Enterococcus, Lactococccus, Lactobacillus Pseudomonas*	---	Ethanol (-60 ^{0}C) for 2-10 min	-45 ^{0}C	<100Pa	4-20 h	Miyamoto-Shinohara et al., 2000; 2006; 2008;
Aerobic	Enterococcus faecalis, Enterococcus durans	---	---	-55 ^{0}C	670 Pa	48h	Carvalho et al., 2003
Aerobic	*Enterococcus faecium, Lactobacillus plantarum*	---	Deep freezing (-80 ^{0}C)	---	3100 Pa	17h	Strasser et al., 2009
--- Data not provided							

Cryo-injury

In freeze drying, freezing is considered to be more detrimental to cells than subsequent drying (Morgan et al., 2006). The process of freezing and, perticularly, the rate of freezing can influence cell viability. Previous studies on preservation discussed that cell injury in slow freezing is mainly due to extra-cellular ice crystal formation and elevated solute concentration (Morgan et al., 2006; Santivarangkna et al., 2008). A slow freezing process creates extra-cellular ice crystals that can damage the fragile cellular components. The lower temperature also leads to concentrated solutes that develop osmotic pressure imbalance resulting in excessive cell fluid leakage. Furthermore, cells can be inactivated due to high solute concentration toxicity (Santivarangkna et al., 2008). In contrast, the major cause of cell injury in rapid freezing is intracellular ice crystal formation (Simione and Brown, 1991; Morgan et al., 2006). The optimum cooling rate results from two opposite effects: when the cooling rate is lower than the optimum, extra-cellular solutes are responsible for the cell injury and when the cooling rate is higher than the optimum, intra-cellular freezing is responsible for cell injury (Berny and Hennebert, 1991). Dumont et al. (2004) discussed that an optimal cooling rate depends on cell type, cell size, water permeability of cell membrane, and type of cell wall (Santivarangkna et al., 2008). Rapid freezing is considered to result in less cell damage due to small ice crystal formation but can creates more difficulty in the subsequent drying process. Whereas slow freezing may impose higher cell damge than rapid freezing due to larger ice crystal formation, but it reduces the drying resistance due to larger empty chanels of larger ice crystals removal (Simione and Brown, 1991).

1.1.6.2   Liquid drying

Microorganisms sensitive to freezing or freeze-drying can be preserved by liquid-drying (Malik 1990a; Malik 1992; Malik 1993; Malik 1998; Malik 1999). In liquid-drying, water associated with the samples is removed from the liquid state to vapor state under vacuum without freezing the samples. It is also called vacuum-drying. Liquid drying is done in two steps; primary drying and secondary drying. At condenser temperatures below -50 ^{0}C, primary drying is performed at 500 to 1000 Pa. The secondary drying is continued under 5 to 10 Pa by maintaining the sample at 20 ^{0}C. Primary and secondary drying times depend on cell suspension volume. For 25 µL cell suspension, the primary drying time was 30 minutes and secondary time was 1 hr, whereas for 30 µL cell suspensions, it was 1 hr and 2 hrs, respectively (Malik, 1990; Malik 1992).

Authors (Malik 1990a; 1992) demonstrated successful preservation of 45 species including various anaerobic phototrophic and other sensitive microorganisms right after liquid-drying and one year of storage at 9 ^{0}C and 2 to 3 years of storages at -30 ^{0}C. Right after drying, the viability of all the species was in between 50 to 100%. Very few species lost their total viability during storage. Some microorganisms which were not strict anaerobes such as *Aquaspirillum arctium* which can grow at 2 to 4 ^{0}C showed better viability after liquid-drying, whereas it was non-viable after freeze-drying. Malik (1993) demonstrated the successful preservation of freshwater unicellular green algae (*Chlorococcales*) using liquid-drying. In this study, all the cultures of *Chlorella pyrenoidosa, Chlorella vulgaris, Selenastrum capricormutum, Scenedesmus subspicatus* and *Euglena gracilis* which are generally used for ecotoxicity testing were viable and stable immediately after drying and after a year of storage at -30 ^{0}C. Also liquid drying resulted in a higher viability when it was compared with freeze-drying.

Malik (1990a) reported problems with liquid-drying which damaged sensitive microorganisms and also induced mutation in the cells in the absence of protective agents. Miyamoto-Shinohara et al. (2006) discussed long-term storage problems with liquid-dried cells. The cell viability of liquid-dried *Saccharomyces cerevisiae* was higher than freeze-dried viability immediately after drying but the decay rate was higher during storage with liquid-dried cultures as compared to freeze-drying cultures. Amino acids have been reported to form both amorphous and crystalline solids when freeze-dried, whereas amino acids only form crystalline solids when liquid-dried and these may not be available to cells during revival. King et al. (1998) compared freeze-drying and controlled low-temperature vacuum drying for long-term preservation of *Lactobacillus acidphilus*. It was found that freeze-drying preserved 80% higher cell viability than vacuum drying for a year of storage at 4 ^{0}C.

1.1.6.3   Thermal drying

Spray drying is typically performed at higher inlet (170 to 200 ^{0}C) and outlet (80 to 105 ^{0}C) air temperatures (Teixeira et al., 1995; To and Etzel, 1997; Gardiner et al., 2000; Desmond et al., 2002; Corcoran et al., 2004; Ananta et al., 2005; Santivarangkna et al., 2007). Instead of inlet temperature, the outlet air temperature is considered to be the major drying parameter affecting the viability of spray-dried cultures (Santivarangkna et al., 2007). Spray drying is widely used to preserve lactic acid bacteria (LAB) in the probiotic industry. Several studies showed the preservation of *Lactobacillus* (Teixeira et al., 1995; To and Etzel, 1997; Gardiner et al., 2000; Desmond et al., 2002; Corcoran et al., 2004; Ananta et al., 2005; Santivarangkna et al., 2007). Teixeira et al. (1995) showed successful preservation of *Lactobacillus bulgaricus* at an outlet air temperature of 80 ^{0}C. Similarly, Desmond et al. (2002) demonstrated the survivability of spray dried

Lactobacillus paracasei NFBC 338 at outlet air temperature of 100 to 105 ^{0}C. Corcoran et al. (2004) showed 25 to 41% survival of spray-dried *Lactobacillus rhamanosus* at an outlet temperature of 85 to 90 ^{0}C in order to obtain RMC less than 4%. Gardiner et al. (2000) preserved *Lactobacillus paracasei* NFBC 338 and *Lactobacillus salivarius* UCC118 using spray drying at an outlet temperature of 80 to 85 ^{0}C. Increased outlet air temperature resulted in decreased cell viability. The survival of spray-dried *L.* paracasei NFBC 338 followed the order of viability of greater than 90% at 70 to 75 ^{0}C with RMC of 7 to 8%, followed by 70% at 80 to 85 °C with RMC 5 to 6 %, and <1% at 100 to 105 ^{0}C with 1% RMC. Ananta et al. (2005) preserved 60% viability of spray-dried *Lactobacillus rhamanosus* GG at an outlet temperature of 80 ^{0}C, and supported the earlier results of increased outlet air temperature resulting in decreased cell viability. To and Etzel (1997) compared the survival of *Lactococcus lactis spp. cremoris* D11, *Lactobacillus casei spp. pseudoplatarum* UL137, and *Streptococcus thermophilus* CH3TH among freezing, freeze drying and spray drying. The percent survival for these species was in the range of 60 to 70 % for freezing and freeze drying, whereas it was 0.5 to 15 % for spray drying at an outlet temperature of 65 to 70 ^{0}C.

Morgen et al. (2006) discussed that fluidized bed-drying at 30 to 40 ^{0}C resulted in better viability and stability of *Penicillium oxalicum* compared to freeze- and spray-drying. It was also mentioned that high cell viability loss of some spray-dried probiotic bacteria was observed during storage. Only one of three lactic acid bacterial strains was found to be viable three months after spray drying. The reasons could be because of high outlet temperatures (80 to 100 ^{0}C) of dried product or a storage condition which might have damaged the cells. Teixeira et al. (1995) showed that spray drying caused cell

membrane damage to *Lactobacillus bulgaricus*. It was observed that spray drying resulted in leakage of cell intracellular components such as cytoplasmic material into the surroundings and also increased cell permeability. The dried cells was also found more sensitive to NaCl and more permeable to *o*-nitrophenyl-β-D-galactopyranoside (ONPG).

Papapostolou et al. (2008) used thermal drying to preserve kefir biomass. Kefir biomass is a known culture of various species of *Kluyveromyces, Candida, Saccharomyces, Pichia* and some lactic acid bacteria of the genus *Lactobacillus.* Three thermal drying methods, (conventional, convective and vacuum drying) were described in this study. In conventional drying, the biomass was baked in a chamber. In convective drying, hot air was used to dry the biomass. In vacuum drying which is also called liquid drying, liquid from biomass was directly removed in vapor form under vacuum without freezing. Drying was performed at three different temperatures, 28, 33 and 38 ^{0}C, and activity or cells for each method was compared in terms of colony-forming units (CFU), lactose fermentation and whey fermentation. In conventional and convective drying, lower lactic acid bacteria viability (CFU/g) was found at 28 and 38 ^{0}C as compared to 33 ^{0}C, whereas in vacuum drying activity increased with increasing temperature. Convective drying at 28 and 33 ^{0}C was resulted in higher lactic acid bacteria viability (CFU/g) as compared to the other methods. Ethanol productivity (g/l-h) was higher with convective dried biomass, whereas lactic acid productivity (g/l-h) was higher with conventional dried biomass.

Thermal injury

In thermal drying especially at high temperature, heat is considered to be the principal cause of cell damage and/or death. Heat stress is identified for denaturing of some crucial

biomolecules such as DNA, RNA, and proteins, also damaging supramolecular assemblies like the cell membrane and ribosomes (van de Guchte et al., 2002). Santivarangkna et al. (2008a) summarized general temperature ranges which affect the different cell components. Temperatures greater than or equal to 65 ^{0}C may deform cell the wall, ribosomes and/or proteins, whereas temperatures below or equal to 64 ^{0}C could damage cellular structures especially the fragile cell membrane. The ribosomes are thought to be the most crucial cellular component for cell inactivation among all other susceptible cell components (Santivarangkna et al., 2008a). Miles (2006) suggested that cells do not die until the functioning copies of critical components goes below the threshold number, and any cell component could be considered as a critical component in this case, if their functioning copy values reach the critical level. However, loss of same critical component may not be necessary under a particular stress (Nguyen et al., 2006).

1.1.7   Protective agent

The survivability of many microorganisms can be improved by adding protective agents to the cell suspension during freezing and/or drying. The protective agents stabilize the integrity of the cell during freezing, drying, storage and rehydration.

The protectant exact mode of action is complex and not completely understood. However, based on various studies which are summarized by others (Leslie et al., 1995; Potts, 2001, Hubalek, 2003; Carvalho et al., 2004; Morgan et al., 2006; Santivarangkna et al., 2008), two protective mechanisms are principally proposed for the cell protection: so called water replacement and vitrification. In the water replacement mechanism, protectant addition replaces cellular water with protectant hydroxyl groups which provide higher stability to cellular membrane and proteins by preventing their deformation. In the

vitrification mechanism, protectant addition vitrifies and creates a physical shield by forming a solid glass matrix around the cell which prevents and/or minimizes the cell membrane damage from ice crystal formation during freezing steps in freeze drying. This dry glassy state provides a physical barrier to O_2 diffusion during drying and subsequent storage and reduces the cell damage occurring due to oxidative stress. This glass matrix also traps the cell's essential nutrient, and fluids/waste products which may leak out due to freezing or drying stress and that could be essential for recovery of cell during rehydration steps. Protective addition helps to maintain osmotic pressure balance and prevent damage to cells during freezing, drying and rehydration processes.

There are various compounds and their derivatives that are used as protective agents for preservation of microorganisms such as sulfoxides, alcohols, saccharides, polysaccharides, amides, imides, peptides, proteins, carbohydrates, and surfactants (Hubalek 2003). These cryoprotectants are either used as individual additives or in combination with one or more additives in certain ratios. Sometime protective agents are called cryoprotectants when they are used for cryopreservation (freezing or freeze-drying).

Cordero and Voltolina (1997) observed active growth of microalgae *Chaetoceros* sp. and *Phaeodactylum tricornutum* in the presence of glycerol and dimethylsulfoxide (Me$_2$SO) as cryoprotectants, whereas no growth was seen in the absence of cryoprotectants after 1 month of storage after slow-rate freezing. King et al. (1998) demonstrated that addition of protective agent during freeze-drying and low-temperature vacuum drying helps to preserve the viability of cells for long-term storage. In this study, 4.15 % glycerol mixed with 10.71% non-fat dry milk was used as a protective agent

during freeze drying and low-temperature vacuum drying of *Lactobacillus acidphilus*.
Results showed that both drying methods achieved more than 80% cell viability with the
addition of protective agent when the culture was stored at 20 ^{0}C and 4 ^{0}C for a year.
Desmond et al. (2002) assessed the protective effect of gum acacia (GA) on the viability
of *Lactobacillus paracasei* after spray-drying and subsequent storage. A 10% (w/v)
concentration of GA as a protectant showed 10-fold higher survival than the control.
Probiotic *Lactobacilli* in GA-containing powders also displayed greater viable recovery
than untreated cultures during storage at 4, 15 and 30 ^{0}C for 4 weeks. Authors (Malik
(1990, Malik, 1990a; Malik, 1992) discussed the protective agents that were effective
during freeze drying to preserve various species of sensitive anaerobic bacteria and were
also found effective during liquid-drying. The order of effectiveness of different
protectants to preserve the cell viability was glutamates, meso-inositol, honey, raffinose,
and adonitol. The author also mentioned that activated charcoal addition (0.01 to 0.1%)
with or without any one of the protectants proved useful to improve cell stability and
viability.

Former studies discussed that the effectiveness of protectants in preserving
microorganisms depends on several factors such as type of microorganisms, cell
concentration, growth medium, process parameters, recovery medium, and storage
condition (Hubalek 2003; Strasser et al., 2009). A single protectant or combination of
protectants for better recoveries is not yet defined and each species is typically
investigated by empirical methods to determine an optimum conditions. Sometime an
increase in cell viability after freezing and/or drying depends on the type of protective
agent used, and its selection is crucial since some protective agents are toxic to different

microbes (Hubalek 2003). The protective agent dimethylsulfoxide (Me$_2$SO) and polyvinylpyrrolidone (PVP) at 10% concentration proved to be effective protectants to provide better viability for methanotrophic bacteria. Methanol at 10% concentration proved to be a more effective protectant for rapid refrigeration (liquid nitrogen) than slow cooling (3 ^{0}C/min) of anaerobic bacteria. Human serum albumin up to 4% concentration was as protective as Me$_2$SO for measles virus frozen at -65 ^{0}C. Ethylene glycol at 4 to 10% concentration was more effective than Me$_2$SO for the cryopreservation of anaerobic rumen fungus. Glucose and sucrose at 10% (median) concentration were frequently used as cryoprotectants for many microorganisms. Sucrose imparted long-term survival to *Bacillus subtitils* and to some other species when frozen with 10% concentration at -10 ^{0}C. The toxicity of an individual protectant is sometimes associated with a temperature, and is an important consideration for particular organisms. The Me$_2$SO as a protectant is less toxic at 0 to 5 ^{0}C and more toxic >5 ^{0}C, whereas PVP as a protectant is less toxic up to room temperatures and more toxic at higher temperatures. Better cell survival was also found to be a function of equilibration time (ET) of protectants with cell suspensions. The optimum ET of cryoprotectants also depends on their penetration rate which is a function of temperature and cell type. For example Me$_2$SO and methanol required l5 min at 4 ^{0}C and 10 to 60 min at 0 to 10 ^{0}C due to quicker penetration, whereas glycerol needed 100 min at 37 ^{0}C for T.*vaginalis* due to its low permeability. Staab and Ely (1987) compared two cryoprotectants to evaluate the viability of 18 strains of pathogenic anaerobes before and after freeze-drying and a year of storage at 5 ^{0}C. These 18 strains included 15 species of the following 8 genera; *Clostridium, Propionibacterium, Bifidobacterium, Eubacterium, Bacteriodes, Fusobacterium, Peptococcus,* and *Peptostreptococcus*.

Sucrose 12% (v/v) as a cryoprotectant showed a higher effectiveness than double-strength skim milk in both conditions; immediately after drying as well as a year storage. Sucrose as a cryoprotectant showed a better effect after a year storage as compared to immediately after drying. All the sucrose-protected freeze-dried strains remained viable at the end of a year of storage, whereas 12 of the 18 freeze-dried strains in double-strength skim milk were nonviable at the end of a year. Immediately after drying, 4 out of 18 freeze-dried strains showed a 55 to 63% reduction in viability on a log scale using double-strength skim milk while no reduction greater than 77% occurring in the sucrose broth medium.

The most frequently used cryoprotectants for bacteria, fungi, algae, protozoa and viruses are Me_2SO, glycerol, sucrose, glucose, skimmed milk and blood serum (Malik 1990; Malik 1992; Hubalek 2003). Previous studies suggested that sugars with higher glass transition temperatures (T_g) may provide a better protective effect during high-temperature drying and/or storage (Santivarangkana et al., 2008; Morgan et al., 2006). T_g is the maximum temperature at which a substance is in its solid glassy state and a subsequent increase in temperature results in the start of crystallization. Sugars with higher viscosity possess higher T_g values. Desmond et al. (2002) used gum acacia, a highly viscous oligosaccharide, to preserve higher viability of a *Lactobacillus* strain that was exposed to high air temperatures (inlet – 170 ^{0}C and outlet – 105 ^{0}C) during spray drying. Santivarangkna et al. (2008) summarized that the protective capacity to stabilize cultures follows the decreasing order of T_g (trehalose 107 ^{0}C > maltose 91 ^{0}C > sucrose 67 ^{0}C > glucose 36 ^{0}C).

The effectiveness of glucose as a protectant was variable with different preservation methods. The protective effect of glucose was evident for freeze drying, but not for the other preservation methods. Strasser et al. (2009) found similar results for glucose and other protective agents when lactic acid bacterial strain *Enterococcus faecium* was freeze- and fluidized-bed dried. The protective effect of glucose was greater for freeze drying than for fluidized bed drying. In contrast, Strasser et al. (2009) found glucose to be inferior to sucrose and trehalose for preserving the viability of freeze- and fluidized-bed-dried lactic acid bacteria stored at 35 ^{0}C for one month.

Higl et al. (2007) demonstrated that cell inactivation rate of *Lactobacillus paracasei* was lower in the glassy state and inactivation was largely affected by the storage temperature and water activity under the non-glassy state. Ohtake et al. (2004) discussed that sometimes the T_g value of sugars is altered by other salts presents in the matrix. The T_g value of trehalose and sucrose in the presence of potassium phosphate increased by 25 and 50 ^{0}C, respectively.

1.1.8 Storage

The shelf life of a dried culture is highly dependent on temperature, oxygen and moisture content. The dried culture is hygroscopic in nature; higher humidity in storage condition increases the moisture content of the dried cells which can accelerate deterotive reaction in dried cells (Santivarangkna et al., 2008a). Similarly, higher RMC or water activity in dried culture led to greater cell viability loss during storage (Aguilera and Karel., 1997). The RMC in the range of 2.5 to 6 % (mass basis) is considered to be optimum value for long shelf life of dried culture during storage (Simione and Brown, 1991; Zayed and Ross, 2004; Gardiner et al., 2002; Desmond et al., 2002). Aguilera and Karel (1997)

suggested RMC values less than 10% (dry mass basis) are typically considered to result in stability during storage. Although low RMC results in higher stability during long-term storage, it may lead to higher cell membrane rigidity which can cause cell leakage during rehydration, resulting in significant cell loss (Santivarangkna et al., 2008a).

Oxygen contact with stored culture has been determined to be the major cause of significant activity loss after simulated long-term storage. Increased oxidation of cell membrane lipids, as measured by changes in the cell membrane fatty acid profiles, has been described to decrease cell membrane fluidity and increase membrane leakage during rehydration (Teixeira et al., 1996; Castro et al., 1996; Borst et al., 2000; Santivarangkna et al., 2008a). Furthermore, free radicals formed during oxidation of biological molecules can lead to significant loss of cell viability or functionality (van de Guchte et al., 2002; Santivarangkna et al., 2008). Authors (Teixeira et al., 1995; Carvalho et al., 2004) suggested that in order to prevent cell inactivation due to oxidative stress and to provide higher cell survivability during storage, dried culture should be stored under vacuum or under controlled water activity.

The first order rate constant (k) of viability loss was dependent on the storage temperature. Higher storage temperature was found to decrease survivability of microorganisms in dried culture (Higl et al., 2007). The storage temperature has a more significant impact on cell survival than the difference between the T_g and the storage temperature (Higl et al., 2007). A storage temperature around 4 to 5 ^{0}C was widely applied for long-term storage of dried culture. Morgan et al. (2006) discussed that storage temperatures of -20 ^{0}C and 20 ^{0}C cause increased loss of cell viability as compared to 4 ^{0}C. Gardiner et al. (2000) reported that cell viability of *Lactobacillus paracasei* NFBC

338 and *Lactobacillus salivarius* UCC118 was inversely proportional to storage temperature. The viability of two months stored cells was higher at 4 ^{0}C compared to 15 and 35 ^{0}C storage. Similar results were observed for *Lactobacillus paracasei* NFBC 338 by Desmond et al. (2002). Authors (Miyamoto-Shinohara et al. 2000; Miyamoto-Shinohara et al. 2006) showed that the survival rate of freeze-dried microorganisms was highly influenced by storage temperature and the degree of vacuum applied. No significant survival loss was found when some species of yeast, gram-positive and gram-negative bacteria were stored for 10 to 20 years at 5 ^{0}C under vacuum. The survival rate of freeze-dried yeast was 97.7% per year, whereas the survival rate for gram-positive and gram-negative bacteria was 96% and 85% per year for 20 years. *Pseudomons* cultures sealed at 6.7 Pa vacuum failed to survive, whereas the same culture sealed under less than 1 Pa vacuum survived for 20 years. One of the explanations was the culture's water activity was extremely low when it was preserved in low vacuum. Malik (1992) showed good viability of all 47 anaerobic phototrophic and other sensitive microbes after liquid drying and during long-term storage at -30 ^{0}C. However, in many cases drastic viability loss was observed during prolonged (> 2 to 3 yrs) storage at relatively higher temperatures (> 9 ^{0}C). The author found that dried cultures sealed in ampoules under vacuum show better survival recoveries during long-term storage (>5 yrs) as compared to the cultures which were unsealed and not under vacuum. However both showed equally good results when stored at -30 ^{0}C or lower temperature. Malik (1988) also showed about 10–100% survival of 36 species of nitrogen-fixing bacteria, 11 species of chemolithorutotrophic bacteria and five species of *Aquaspirillum* after freeze drying and during 2–3 years of storage at 9°C. Desmond *et al.* (2002) demonstrated the cell viability

of spray-dried *Lactobacillus paracasei* increased with increased temperatures for the first four weeks and then decreased with increased temperatures and was almost negligible after 8 weeks when the culture was stored at 15 and 30 ^{0}C compared to 4 ^{0}C. The increase in cell viability during the first four weeks was due to decreased water activity with increasing storage temperatures. King et al. (1998) demonstrated freeze-dried *Lactobacillus acidphilus* stored at 4 ^{0}C showed 98% higher survival after a year as compared to culture stored at 20 ^{0}C. The study concluded that the cell viability during storage was successfully predicted using an accelerated storage test method by means of an Arrhenius relationship between cell deterioration rate and storage temperature. Sakane and Kuroshima (1997) demonstrated that accelerated storage of liquid-dried bacterial cultures for 2 weeks at 37 ^{0}C can simulate 20 years of storage at 5 ^{0}C under vacuum. In this study, the authors addressed the linear correlation between the survival rates of 59 liquid-dried bacterial cultures after 21 to 24 years of storage at 5 ^{0}C under vacuum, with the correlation coefficient 0.95 with the accelerated storage. The viability of the cultures decreased linearly in the early stage of preservation and became stable during storage for 6-8 years and then was subsequently unchanged during the next 16 years. This approach was applied by Iino and Suzuki (2006) to study the long-term storage of pure methanogenic cultures. Ananta et al. (2005) found higher cell viability for stored *Lactobacillus rhamanosus* at 37 ^{0}C than at 25 ^{0}C which contradicted the general observation of lower cell viability of preserved culture at higher storage temperature.

1.1.9 Rehydration

Dried microorganisms are revived in the step of rehydration and it is considered to be one of the crucial steps for cell survivability since dried cells already underwent sublethal injury due to drying and/or storage and rehydration in inappropriate conditions may not

be helpful to recover from cell damage (Costa et al. 2000). The rehydration media in terms of its osmolarity, pH and nutrients as well as its temperature, time and volume can significantly affect the recovery rate and survivability of cells (Carvalho et al., 2004, Morgan et al., 2006). Morgan et al. (2006) summarized in their review article that the same rehydration solution as that used as protective medium increases cell viability after rehydration. For example, using 10% skim milk as a protectant and as a rehydration medium for freeze drying of *Candida sake* cells improved the survival of the cells. The complex medium which provides a high osmotic pressure to the freeze-dried cells resulted in better recovery during the rehydration process by controlling rehydration rate and minimizing an osmotic shock (Costa et al., 2000; Carvalho et al., 2004). Complex rehydration media may assist in repair of the injured cells by providing additional nutrients and essential cell components required to repair damaged cells (Morgan et al., 2006). It was also observed that rehydration temperature and rehydration time also influence cell recovery after freeze drying. The effect of rehydration temperature on cell viability could be due the higher rehydration temperature than cell T_m may prevents recovery of cell membrane transition from gel to liquid which resulted from the drying stress (Potts, 2001; Santivarangkna et al., 2008). Chipley and May (1968) discussed that higher reconstitution temperature was inversely proportional to cell viability. In this study, freeze dried chicken meat with some aerobic and anaerobic bacteria was rehydrated at 100 ^{0}C, 85 ^{0}C, 55 ^{0}C and at room temperature (20 to 23 ^{0}C). At room temperature, many genera of aerobes, anaerobes and facultative anaerobes exhibited higher viable cell count and it decreased with increasing temperature. Only spore-forming bacteria survived rehydration at 85 and 100 ^{0}C. The rehydration of S. *cerevisiae* at 15-25

^{0}C produced higher viable recovery as compared to 35-45 ^{0}C, whereas slow rehydration

(7-16 days) under controlled conditions increased the survival rate as compared to

immediate rehydration (Morgan et al., 2006). In contrast to the above studies, Teixeira et

al. (1995) reported a linear increased in cell survivability of *Lactobacillus bulgaricus*

with increased rehydration temperature between 4 ^{0}C to 50 ^{0}C in ambient conditions. A

significant difference was found when the cells were rapidly or slowly rehydrated and

slower rehydration increased the cell survival.

1.2   Methanogen tolerance to drying, O_2 and heat

Cleland et al. (2004) successfully preserved the viability of *Methanothermobacter*

thermautotrophicus after freeze drying performed under strictly anaerobic conditions.

Castro et al. (2002) determined the methanogenic activity of biomass obtained from an

anaerobic lagoon after freeze drying under strictly anaerobic conditions. The percent

specific methanogenic activity of freeze-dried anaerobic biomass was approximately 18%

for glucose and 35% for acetate after two months of storage, whereas it was 15 and 20%

after five months of storage, respectively. Furthermore, addition of trehalose and glycerol

as protective agents preserved higher methanogenic activity of biomass after freeze-

drying compared to biomass freeze-dried without protective agents, and trehalose (10%

v/v) was found to be more effective to preserve methanogenic activity than glycerol. A

similar study performed by Colleran et al. (1992) showed anaerobic biomass freeze-dried

under strict anaerobic conditions maintained 13 to 85 % of specific methanogenic activity

after drying and 0 to 54% activity after drying and seven months of storage at 4 °C for

acetate, butyrate and H_2:CO_2 substrates in the presence of different cryoprotectants. A

10% glucose addition to granular anaerobic biomass resulted in 32% higher

hydrogenotrophic methanogenic activity after freeze drying and 49% higher activity after seven months of storage at 4 ^{0}C under anaerobic conditions (Colleran et al., 1992).

Iino and Suzuki (2006) preserved significant viability as well as methanogenic activity of *Methanobrevibacter arboriphilus* NBRC 101200, *Methanoculleus chikugoensis* NBRC 101202, *Methanosarcina mazei* NBRC 101201 and *Methanothermobacter thermautotrophicus* NBRC 100331 using liquid drying. The viable cell number of the preserved cultures was between 10^4 to 10^6 cells/mL compared to 10^5 to 10^6 cells/mL in non-dried culture. The percent methanogenic activity of the preserved cultures was in the range of 25 to 45 % immediate after drying, after one week of storage at 4 ^{0}C and after simulated long-term storage using 37 ^{0}C for two weeks under vacuum.

Kendrick and Kral (2006) demonstrated survival of *Methanosarcina barkeri, Methanobacterium formicium* and *Methanothermobacter wolfeii* grown on simulated Mars soils when dried in a desiccator containing anhydrous calcium sulfate under strict anaerobic conditions. *M.barkeri* survived drying for 10 days, while *M. formicium* and *M. wolfeii* were able to survive for 25 days which was assayed by measuring methane production rate over time. In a similar study, Karl et al. (2011) analyzed the effect of different pressures on desiccation. The results illustrated that *M.barkeri* survived 330 days of drying at 1 bar whereas *M. formicium* and *M. wolfeii* survived 120 and 180 days, respectively. At a lower pressure of 6 mbar, all the strains survived approximate 120 days of drying.

Methanogens are obligate anaerobes and even a trace amount of O_2 is considered to be lethal. Oxidative stress caused by reactive O_2 species (ROS) can significantly damages the cell membrane, crucial proteins and enzymes more than the resulting higher

redox potential that inhibits methanogenesis and growth (Jarrell, 1985; Fetzer et al., 1993; Brioukhanov and Netrusov, 2007; Liu et al., 2008; Yuan et al., 2009). Previous studies showed that the F-420-hydrogenase enzyme complex inactivates in the presence of O_2 which is an important complex produced by methanogens during methanogenesis (Schonheit et al., 1981; Jarrell, 1985).

Authors (Brioukhanov and Netrusov, 2007; Botheju and Bakke, 2011) discussed limited tolerance of some methanogens to O_2, including *Methanosaeta concilii* (formerly known as *Methanothrix soehngenii*), *Methanosarcina barkeri*, *Methanobacterium bryantii*, *Methanothermobacter thermoautotrophicus,* and *Methanobrevibacter arboriphilus*. In contrast, other methanogens, such as members of the genus *Methanococcus*, are reported to be more oxygen sensitive. Jarrell (1985) correlated the higher tolerance of methanogens with their ability to produce detoxification enzymes such as superoxide dismutase and catalase. Liu et al. (2008) discussed that methanogenic genera *Methanobacterium, Methanosarcina*, and *Methanobrevibacter* all produce these detoxification enzymes and were observed to survive desiccation in the presence of O_2 where as *Methanococcus, Methanoplanus,* and *Methanoculleus* did not survive desiccation in the presence of O_2. Studies estimating CH_4 emission from several dried upland soils including hot desert soils and dried paddy soils suggested methanogens survival in seasonal oxic exposure (Angel et al., 2011; Angel et al., 2012)

Ueki et al. (1997) demonstrated the heat tolerance of methanogens by measuring the CH_4 production in air-dried paddy soil heated at $100\ ^0C$ in an oven for 2 hrs. A similar study was performed by Tryon and Kral (2010), who discussed the heat tolerance of *Methanothermobacter wolfeii* and *Methanosarcina barkeri* from simulated Mars soils

dried at 100 ^{0}C for one minute to one hr. The results indicated survival of both

methanogens and *M. barkeri* survived longer than *M. wolfeii*.

1.3 Bioaugmentation in anaerobic biotechnology

Bioaugmentation is defined as the addition of specific microorganisms or enzymes to a

biological system to enhance the process (Deflaun and Steffan, 2002; Rittmann and

Whiteman, 1994). It can also be viewed as the use of a preselected specialized, mutant,

adapted mixed culture that can assist to stabilize and improve the overall process

performance at lesser cost (Nyer and Bourgeois, 1980). Bioaugmentation effectiveness

and proper implementation is a function of a system's environment and a substrate's

characteristics. Efforts have been made to see the bioaugmentation effect on specific

organic chemical removal, fats, oil and grease removal, cellulose degradation, odor

reduction and stressed digester recovery during anaerobic treatment.

1.3.1 Bioaugmentation for difficult-to-degrade organic compounds

Horber et al. (1998) investigated the bioaugmetation effect of a strictly anaerobic,

reductively dechlorinating bacterium, *Dehalospirillum multivorans* on dechlorination of

perchloroethylene (PCE) in UASB reactors. The test reactor bioaugmented with D.

multivorans converted 93% of the PCE to dichloroethene (DCE), whereas the non-

bioaugmented reactor with the same amount of active biomass converted only 43% of the

PCE to dichloroethene. Also the control reactor containing pre-sterilized granular

biomass supplied with D. *multivorans* showed 93% of PCE reduction to DCE. It was also

observed that the test reactor and control reactor showed PCE to DCE conversion at

hydraulic retention times (HRTs) much lower than the reciprocal maximum growth of D.

multivorans, indicating the microbes were immobilized in the active and pre-sterilized

biomass granules. Charest et al. (1999) presented higher removal efficiency of phenol

(97%) and o-cresol (83%) from the petrochemical refinery effluent when bioaugmented the fixed film anaerobic reactor with a methanogenic consortium compared to normal operating reactor. Similarly, results were reported by Hajji et al. (1999) and Hajji et al. (2000).

Cirne et al. (2006) observed an increased in methane production rate due to bioaugmentation using a lipolytic bacterial strain, *Clostridium lundense* (DSM 17049[T]), in anaerobic digestion of lipid-rich waste (10% lipids triolein).

Nielsen et al. (2007) demonstrated improved recovery of methane production from the degradation of manure, the liquid fraction of manure, raw fibers from the manure and anerobically digested fibers when bioaugmented with two thermophilc strains, *Caldicellulosiruptor lactoaceticus* (strain 6A) and *Dictyoglomus* (strain B4a). The batch study results showed that strain 6A yielded more methane compared to strain B4a. When strain 6A was further used for bioaugmenting a two-stage thermophilic digester ($68\ ^0C/55\ ^0C$), 93% more methane was yielded from the first stage reactor, and overall methane yield was slightly improved. It was also observed that the increase in methane production lasted up to the 47th day of the test and, at the 50th day the methane production suddenly decreased which was indicative of wash-out of strain 6A from the reactor.

Angelidaki and Ahring (2000) reported 30% higher methane production by the treatment of recalcitrant matter present in the manure when it was bioaugmented by the hemicellulose-degrading bacterium B4 compared to the control reactors.

1.3.2 Bioaugmentation for odor reduction

Duran et al. (2006) concluded that bioaugmenting anaerobic biosolids with commercial microbes *Bacillus, Pseudomonas* and *Actinomycetes* improved methanogenesis and

reduced odor during digestion and dewatered biosolids storage. The bioaugmented

digester generated 29% more net methane and 54% less residual propionic acid

concentration than a control. In addition, a bioaugmented digester produced odorous

methyl mercaptan (CH_3SH) in only trace concentration, whereas a CH_3SH concentration

of about 300 ppm_v was found in the control digester during 10 days of post-digested

biosolids storage. Similary, peak dimethyl sulfide (CH_3SCH_3) produced by the stored

biosolids from the bioaugmented digester was only 37% of that from the control. A low

concentration of CH_3SH and CH_3SCH_3 in the bioaugmented reactor resulted in less odor.

1.3.3 Bioaugmentation for accelerating the reactor start-up

Saravananane et al. (2001) accelerated the start-up of anaerobic fluidized-bed reactor

seeded with methanogenically active biomass adapted on pharmaceutical industrial

wastewater compared to non-adapted biomass. The start-up time was reduced by 8 days

due to bioaugmentation of methanogenically active adapted biomass compared to non-

adapted biomass to achieve COD removal of 88% in the digesters. Guiot et al. (2000)

reported that bioaugmentation of 2 to 5% (mass/mass of non-acclimated biomass) of

enriched methanogenic mixed culture considerably decreased the start-up period of

UASB digesters when different bioaugmentation dosing of enriched methanogenic

culture was used to degrade phenol, ortho-and para-cresol. The results showed at least

two fold higher degradation of the targeted compounds in a bioaugmented digesters

compared to non-bioaugmented control digesters during continuous operation.

1.3.4 Bioaugmentation for shock load upset

O' Flaherty et al. (1999) and O' Flaherty and Colleran (1999) achieved stressed digester

recovery of high sulphate concentration by bioaugmenting a hybrid digester with 25

gVSS/L of sulphate-adapted biomass. The hybrid digester removed less than 60% COD

and received 4 g/L sulphate in the influent, whereas the control digester receiving no

sulphate and removed 95% COD. With successful bioaugmentation, improvement in the

COD removal was observed after one retention time (48 hrs) and the COD removal

exceeded 95%. Bioaugmetation might have created favorable conditions for propionate-

and H_2 utilizing sulphate reducing bacteria in the biofilm section. Lynch et al. (1987)

used bioaugmentation culture composed of anaerobic biomass and pure culture of

Methanosarcina barkeri to improve the performance of organically overloaded digester.

The results showed a higher rate of volatile organic acids degradation and shorter

recovery time for bioaugmented digesters compared to non-bioaugmented digesters. Tale

et al. (2010) recovered organically overloaded digesters by bioaugmenting with a

propionate enriched culture. The digesters were shocked by 12 times higher COD than its

daily feed. Bioaugmented digesters recovered 25 days sooner than non-bioaugmented

digesters. The methane production in bioaugmented digesters recovered 28 days earlier

than in non-bioaugmented digesters. The influence of bioaugmentation persisted more

than 15 SRTs for enhanced SCOD removal and more than 12 SRTs for methane

production.

1.3.5 Bioaugmentation for toxic upset

Schauer-Gimenez et al. (2010) proved that bioaugmentation using a H_2-utilizing

methanogenic culture improved recovery of anaerobic digesters after toxicant exposure.

The digesters were exposed to O_2 for a week. The average SCOD in bioaugmented

digesters declined to 600 mg/L in 2.5 months while non-bioaugmented digesters couldn't

reduce the SCOD to 600 mg/L even after 5 months. The propionate concentration in

bioaugmented digesters decreased from 1500 mg/L to 60 mg/L in 10 SRTs, whereas in

non-bioaugmented digesters it never decreased below 60 mg/L even in 15 SRTs. The

average methane production in bioaugmented digesters was approximately 60% higher

than that of the non-bioaugmented digesters.

1.3.6   Bioaugmentation for psychrophilic digester

Qiu et al. (2011) demonstrated 40% and 17% increased in biogas production from pig

and cow manure respectively when enriched psychroactive methanogenic consortia was

bioaugmented in psychrophilically (15 ^{0}C) operated anaerobic digesters.

1.4   References

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Chapter 2 Methanogens Endurance to Drying in Presence of Air under Extreme Temperature

2.1   Introduction

Methanogenic microorganisms are in the archaeal domain, play a significant role in the Earth's carbon cycle and have been found in almost all anoxic environments. Methanogens produce CH_4 biologically. "*CH_4 is the third most prominent greenhouse gas on the Earth after water vapor and CO_2 and has 25 to 41 times the global warming potential of CO_2*" (Forster et al., 2007; Shindell et al., 2009). Almost 90% of the Earth's atmospheric CH_4 is attributed to biological activity with two-third of this coming from wetland soils (Megonigal and Guenther, 2008; Conrad, 2007).

Biological CH_4 production is also of interest to astrobiologists studying the possibility of life on Mars due to the recent discovery of CH_4 in the Martian atmosphere (Formisano et al., 2004; Krasnopolsky et al., 2004; Mumma et al., 2009) and the possible existence of liquid water on the surface and/or subsurface of Mars (Squyres et al., 2006; Andrews-Hanna et al., 2007). However, any life on a Mars has been hypothesized to require the ability to survive under extreme conditions of dry soil, high temperature and pressure variation above and under the soil surface (Atreya et al., 2007; Levin and Straat, 2009).

Along with environmental and astrobiological importance, biological methanogenesis also has practical importance due to its cost effective treatment of various organic pollutants. For example, methanogenic biotechnology has been used to treat organic wastes and wastewaters generated from municipal (Seghezzo et al., 1998), and various agricultural industries (Cieck, 2003; Ward et al., 2008), leachate generated at landfill sites (Timur and Ozturk, 1999; Renou et al., 2008), and certain toxic wastes

including halogenated and aromatic organic compounds from groundwater (Haggblom et al., 2002; Guiot et al., 2010). Variation of environmental and operational parameters often upset the biological methanogenesis process in treatment systems (Leitao, et al., 2006). More resilient methanogenic communities would improve system stability and performance. An understanding of what constitutes a more resilient methanogenic community is being developed to understand and improve engineering processes (McKeown et al., 2009; Morris, 2011) and develop applications of these communities to enhance the stability and performance of engineered systems (Schauer-Gimenez et al., 2010; Tale et al., 2011).

Possibilities lie in the future development of beneficial and/or application-specific methanogenic cultures. When developed, their preservation via drying should be considered to yield a cost effective option for research and practical applications. This is similar to the current preservation approach of lactic acid bacteria for human probiotics (i.e., yogurt) that uses well-established high temperature spray drying operated at 80 to 105 ^{0}C and ultra-low temperature freeze drying (To and Etzel., 1997; Corcoran et al., 2004; Wang et al., 2004; Morgan et al., 2006; Santivarangkna et al., 2007). To apply these preservation methods to methanogenic cultures, tolerance of methanogens to theses methods is crucial. Furthermore, knowledge of methanogen tolerance to O_2 could be helpful to optimize preserving, handling and storing cultures in the presence of air during large-scale production.

Methanogens are obligate anaerobes, and even a trace amount of O_2 is considered to be lethal. It is molecular O_2 which significantly damages the cell membrane, crucial proteins and enzymes more than the resulting higher redox potential that inhibits

methanogenesis and growth (Jarrell, 1985; Fetzer et al., 1993; Brioukhanov and Netrusov, 2007; Liu et al., 2008). Others have shown that the F-420-hydrogenase enzyme complex inactivates in the presence of O_2, and it is an essential complex produced by methanogens during methanogenesis (Schonheit et al., 1981; Jarrell, 1985). Although, a few extremophile methanogens can survive at unusually high temperatures such as geothermal environments above 80 ^{0}C or ultra-low temperature arctic soils (Cavicchioli, 2006), these conditions are ostensibly lethal to the majority of known methanogenic species. Santivarngkna et al., (2008a) discussed temperatures greater than 65^{0}C can lead to ribosomal, protein and membrane damage which causes cell death. In bio-hydrogen production studies, heat treatment at 80-104 ^{0}C for 15 minutes to 2 hrs has been employed to inactivate methanogens in anaerobic inocula to stop methanogenesis and, thus, increase hydrogen production (Oh et al., 2003; Zhu and Beland 2006; Kraemer and Bagley; 2007). Similarly, freezing at ultra-low temperature exerts severe cryo-injuries to critical cell components by forming ice crystals within and around the cells causing cell death after thawing (Morgan et al., 2006; Santivarngkna et al., 2008; Santivarngkna et al., 2008a). Drying imposes severe stress on the cell by removing water molecules associated with cellular components which causes a deformation to the cell membrane and crucial cellular proteins, leading to significant loss in cell survivability (Leslie et al., 1995; Pott, 2001). The damage to the cell is higher for drying methods due to higher rate drying using compared to the lower rate drying of natural desiccation (Fetzer et al., 1993; Santivarngkna et al., 2007).

Contradictory to the above findings, recent studies have shown limited tolerance of some methanogens to harsh conditions. Studies revealed that some methanogens have

the ability to produce O_2 detoxifying enzymes as a defensive mechanism to oxidative stress (Brioukhanov, et al., 2002; Brioukhanov et al., 2006; Kratzer et al., 2011). Other researchers reported methanogens possess limited tolerance to micro-oxygenation (Zitomer, 1998; Zitomer and Shrout, 1998; Botheju and Bakke, 2011). Studies estimating the CH_4 emission from several dried upland soils including hot desert soils, and dried paddy soils describe methanogen tolerance to desiccation and O_2 and revival of methanogenic activity after return to seasonal anoxic conditions (Angel et al., 2011; Angel et al., 2012). Laboratories studying methanogens as model organisms for life on Mars reported methanogen isolate can grow on dried simulated Mars soils and methanogen tolerance to natural desiccation and high pressure (1bar) desiccation under strict anaerobic conditions (Kral et al., 2004; Kendrick and Kral, 2006; Kral et al., 2011). The authors briefly discussed CH_4 production in pre-dried soils heated at 100 ^{0}C for 1 min to 2 hrs under strict anaerobic conditions (Ueki et al., 1997; Tryon and Kral, 2010). A few studies have demonstrated preservation of methanogenic culture activity using freeze and liquid drying under strict anaerobic conditions (Colleran et al., 1992; Castro et al., 2002; Iino and Suzuki, 2006).

Overall, most available literature contradicts the opinion that methanogens are tolerant to O_2, temperature extremes and drying. Few studies have shown the limited tolerance of some methanogens to these conditions. Very limited research has investigated the combined effect O_2, temperature and drying on methanogens. Based on the available literature, we assumed that the cumulative effect of these conditions would be highly stressful to methanogen survival. With the multidisciplinary interest in methanogen endurance and survival in harsh environments, we wanted to determine the

tolerance of mesophilic methanogenic cultures to the combined effect of drying, extreme temperature, and air. Specifically, we investigated heat drying methanogens at 104 ^{0}C in the air for 12hrs, and by freezing them at -196 ^{0}C in the air and subsequent drying at subzero temperature.

2.2   Methods

2.2.1   Culture and medium

A mixed methanogenic culture (MMC) was obtained from a 15-L anaerobic, completely mixed stirred tank reactor (CSTR) fed non-fat dry milk (Roundy's Inc, Milwaukee, WI, USA) at a 2.7 gCOD/L-d loading and basal nutrient medium operated at 35 $\pm$2 ^{0}C over 3 years. The non-fat dry milk contained the following per 100 g of material [g]: carbohydrates [52], protein [35], sodium [0.05], and potassium [0.2]. The basal nutrient medium was similar ot that described by Speece (2008) and contained the following [mg/L]: NH_4Cl [400]; $MgSO_4 \bullet 6H_2O$ [250]; KCl [400]; $CaCl_2 \bullet 2H_2O$ [120]; $(NH_4)_2HPO_4$ [80]; $FeCl_3 \bullet 6H_2O$ [55]; $CoCl_2 \bullet 6H_2O$ [10]; KI [10]; yeast extract [100]; the trace metal salts $MnCl_2 \bullet 4H_2O$, NH_4VO_3, $CuCl_2 \bullet 2H_2O$, $Zn(C_2H_3O_2)_2 \bullet 2H_2O$, $AlCl_3 \bullet 6H_2O$, $NaMoO_4 \bullet 2H_2O$, H_3BO_3, $NiCl_2 \bullet 6H_2O$, $NaWO_4 \bullet 2H_2O$, and Na_2SeO_3) [each at 0.5]; $NaHCO_3$ [5000]; and resazurin [1].

2.2.2   Culture thickening and drying

Biomass concentration was measured in the form of volatile suspended solids (VSS) using standard methods (APHA et al., 2005). Culture liquid with biomass concentrations of 3.5 to 4 gVSS/L was concentrated from 300 mL to 20 mL by centrifugation at 4500 rpm (2.5 gravitational force) for 10 min prior to drying. For heat drying, an aliquot of concentrated culture was transferred to a 25-mL porcelain crucible and dried in an oven (Model 1350GM, Sheldon Manufacturing Inc., Cornelius, OR, USA) at 104 ^{0}C for 12 hrs

in the presence of air. For freeze drying, concentrated culture was transferred in the presence of air to a freeze-drying flask and shell frozen in liquid nitrogen by swirling the flask for 7 min. The frozen culture was then dried for 24 hrs using a bench-top freeze dryer (3GenMP Opti-Dry, Millrock Technology, Kingston, NY, USA) under vacuum at 13.3Pa pressure and a condenser temperature of -45 ^{0}C (Staab and Ely, 1987; Probucan 1991; Simione and Brown, 1991).

2.2.3 Specific methanogenic activity (SMA) testing

Specific methanogenic activity (SMA) testing was conducted for H_2 and acetate as described in detailed in following Chapter 3 based on the protocol suggested by others (Coates et al., 1996; Anagelidaki et al., 2007). Briefly, the preserved culture was reconstituted in basal nutrient medium containing L-cysteine hydrochloride (500 mg/L) as a reducing agent (Coates et al., 1996), and 25 mL of suspension was added to 160 mL serum bottles. All assay bottles were prepared in triplicate, sealed after sparging with O_2-free gas (N_2:CO_2, gas mix 3:1v/v), and incubated at 35 ^{0}C and 150 rpm in an incubator (C25KC, New Brunswick Scientific, Edison, NJ, USA). The endogenous biogas produced was released during the first 48 hrs, and then substrate (either H_2 or calcium acetate) was added to test bottles. For the H_2 assay, 100 mL of H_2:CO_2 gas mix (4:1 v/v) was pressurized into test bottles containing biomass VSS concentration ≤ 0.3 g VSS/L. For the acetate assay, 10 g/L of calcium acetate was introduced to test bottles maintaining biomass VSS concentration ≤ 2.0 g VSS/L. The controls were run without adding the substrates to correct the net CH_4 production of test bottles. The SMA was estimated by measuring the maximum CH_4 production rate divided by total VSS mass in the bottles. The methanogenic activity of non-dried culture was determined using a similar protocol

as discussed above except no reconstitution step was required. The activity lag was
calculated as the time after feeding required to obtain the maximum CH_4 production rate.

2.2.4   Microbial community identification and quantification
2.2.4.1   Methanogenic community identification using *mcrA* gene-specific primer
Nucleic acid extraction: The DNA was extracted from non-dried culture using
Powersoil[TM] DNA Extraction Kit (MOBIO, Carlsbad, CA, USA) according to
manufacture's alternative lysis protocol for minimizing DNA shearing during extraction.
DNA was analyzed for purity on agarose gels (1.5% w/v) stained with ethidium bromide
and quantified using a spectrophotometer (Nanodrop ND-1000, ThermoScientific,
Waltham, MA, USA). Gels were visualized using a transilluminator (M-20, UVP,
Upland, CA, USA) with the help of Lab Works software (v.4.6.00.0).

Polymerase Chain Reaction (PCR): PCR was performed on purified DNA of
non-dried culture using *mcrA* gene-specific primers (Luton et al., 2002) (Table 2.1). The
PCR reaction mixture (50 µL) contained 100 ng template, 0.1 µM each primer, 0.2 µM
dNTPs, 1X Colorless GoTaq Reaction Buffer with1.5 µM $MgCl_2$ (Promega, Madison,
WI, USA), and 1.25U GoTaq polymerase. The PCR conditions were as follows: 95 ^{0}C for
5 min, 35 cycles of 95 ^{0}C for 1 min, 49 ^{0}C for 1 min, and 72 ^{0}C for 3 min, and a final
extension of 10 minutes at 72 ^{0}C (Morris, 2011).

Cloning: Clones were developed by ligating the *mcrA* PCR products into the pCR
2.1-TOPO® plasmid vector of the TA cloning kit and transformed into One Shot
TOP10[TM] chemically competent *E.coli* as per the manufacturer's guide (Invitrogen,
Carlsbad, CA, USA). Randomly screened white colonies were used directly in PCR
reactions using PUC primers (PUCF-5´-GTAAAACGACGGCCAG and PUCR-5´-

CAGGAAACAGCTATGAC) to generate *mcrA* DNA sequences for restriction fragment

length polymorphism (RFLP) analysis and sequencing. The PCR reaction mixture (50

µL) was run at 94 ^{0}C for 1 min, 55 ^{0}C for 1 min, 72 ^{0}C for 1 min, and a final extension

of 10 minutes at 72°C (Morris, 2011).

RFLP: PUC-amplified PCR product from individual clone containing *mcrA* was

digested using the restriction enzymes *Taq^{α}I*, *RsaI*, and *Sau96I* (New England Biolabs,

Ipswich, MA, USA) as described in the manufacturer's manual. Digested products were

separated on 2% (w/v) agarose gel and visualized by ethidium bromide staining as

discussed above.

DNA sequencing: Unique clones determined from RFLP methods were purified

using Qiaquick™ PCR Purification Kit (Qiagen, Valencia, CA, USA) as per the

manufacturer's instructions and sequenced at the University of Chicago Cancer Research

Center DNA Sequencing Facility. FinchTV (Geospira Inc., Seattle, WA, USA) and

VectorNTI (Invitrogen Corporation, Carlsbad, CA, USA) software was employed to

analyze forward and reverse sequences. Consensus sequences were assembled using the

ContigExpress tool in VectorNTI software and vector sequences were eliminated with the

help of VacScreen program in the Basic Local Alignment Search Tools (BLAST).

Nucleotide matching of *mcrA* sequences was performed using the BLAST search engine

to identify similar reference *mcrA* sequences in GenBank$^®$.

Phylogenetic analysis: The consensus and reference sequences were aligned

using the ClustalW bioinformatics tool described elsewhere (Thompson et al., 2002). A

phylogenic analysis was performed based on the distance matrix algorithm using the

Phylogeny Inference Package (PHYLIP) dnadist programs (Felsenstein, 2005). A

bootstrap analysis was conducted using the SEQBOOT program found in the PHYLIP

suite of programs. A total of 100 possible bootstrap samples were generated and analyzed

using the PHYLIP algorithm to construct final consensus neighbor-joining and maximum

likelihood trees which were further visualized using FigTree v1.2.3. The resulting trees

were found to be similar and the neighbor-joining tree is presented herein.

Sequence accession number: The sequences generated from the clone library

were submitted to GenBank® under accession numbers JN9380-JN93943.

2.2.4.2 Methanogenic community quantification using *mcrA* gene-specific and *16S rRNA* genus-specific primers

Nucleic acid extraction: DNA and RNA were extracted from non-dried and dried

samples using the RNA PowerSoil^{TM} Total RNA Isolation Kit and DNA Elution

Accessory Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) as per the

manufacturer's protocol. Extracted RNA was treated with RNase-free DNase (Qiagen,

Valencia, CA, USA). RNA and DNA were purified using an RNeasy Mini Kit (Qiagen,

Valencia, CA, USA) and DNA Ultra Clean Kit (MO BIO Laboratories Inc., Carlsbad,

CA, USA), respectively. RNA and DNA were analyzed on agarose gels (1.5% w/v) and

quantified as discussed above.

ReverseTranscriptase-PCR (RT-PCR): Extracted single-stranded RNA was

reverse transcribed to complimentary DNA (cDNA) using the iScript® Select cDNA

Synthesis Kit (Biorad, Hercules, CA, USA). A sample of each RNA extract (1200 ng)

was used as a template for RT-PCR using the *mcrA* reverse primer designed by Luton et

al. (2002) and 800 ng of RNA was used as a template for16S rRNA 1000R designed by

Gantner et al. (2011). No-template and no-reverse-transcriptase controls were also

included for each sample.

Quantitative PCR (qPCR): DNA concentration of 1ng/µL and cDNA concentration of 40ng/µL were standardized for all qPCR reactions. The qPCR was performed with the *mcrA* gene-specific primers and the *16S rRNA* genus-specific primers for *Methanospirillum, Methanoculleus, Methanosaeta* and *Methanosarcina* that are listed in Table 1. A standard curve was developed with each run and *mcrA* and *16S rRNA* gene and transcript copies of the samples were estimated with respect to the standard curve. The protocol used for standard curve development was described in detail elsewhere (Morris, 2011). A no-template control was also included and a dissociation curve was performed to check product specificity. Products from initial runs were examined for specificity using 1.5% (w/v) agarose gels. The final qPCR mix per 25 µl reaction was as follows: 1X iQ™ SYBR® Green Supermix reaction buffer containing dNTPS , iTaq DNA polymerase and 3 mM $MgCl_2$ (Biorad, Hercules, CA, USA); and template DNA (1ng). The *mcrA* and genus specific primers were used at a final concentration of 750 nmol/L and 300 nmol/L, respectively. A Biorad My iQ single color RT-PCR detection system was employed in real-time PCR reactions. Thermal cycling comprised of an initial denaturation at 95^0C for 10 min, followed by 45 cycles of $95\,^0C$ for 30 s, 58.5 to 65 ^0C for 60 s and a final extension at $72\,^0C$ for 7 min. Annealing temperatures of $58.5\,^0C$ were used for *mcrA*, $60\,^0C$ for *Methanospirillum* and M*ethanosaeta*, and $65\,^0C$ for M*ethanoculleus and Methanosarcina.* The amplification program was followed by a denaturation curve program (80 cycles 10 sec in length starting at $55\,^0C$ and increasing in $0.5\,^0C$ increments) to check for product specificity. Products from initial runs were also examined for specificity using 1.5% (w/v) agarose gels as described above. Starting

quantity amounts and threshold cycle values were calculated using the MyiQ™ optical

system software version 1.0.

2.2.5 Analysis

The transcript and gene copies were estimated using the formula discussed elsewhere (Yu

et al., 2005) and modified with regards to culture's biomass concentration.

Transcript copies/gVSS

$$= \frac{\text{RNA concentration } \left(\frac{\text{gRNA}}{\text{mL}}\right) \times 6 \times 10^{23} \left(\frac{\text{copy}}{\text{mole}}\right)}{\text{Biomass concentration } \left(\frac{\text{gVSS}}{\text{mL}}\right) \times \text{amplicon size (bp)} \times 330 \left(\text{g} \frac{\frac{\text{RNA}}{\text{mole}}}{\text{bp}}\right)} \ldots \text{Eqn 1.}$$

Gene copies/gVSS

$$= \frac{\text{DNA concentration } \left(\frac{\text{gDNA}}{\text{mL}}\right) \times 6 \times 10^{23} \left(\frac{\text{copy}}{\text{mole}}\right)}{\text{Biomass concentration } \left(\frac{\text{gVSS}}{\text{mL}}\right) \times \text{amplicon size (bp)} \times 660 \left(\text{g} \frac{\frac{\text{DNA}}{\text{mole}}}{\text{bp}}\right)} \ldots \text{Eqn 2.}$$

A one sided Student t-test with unequal population variance was used to compare

methanogenic activity and qPCR results.

Table 2.1. Methanogenic gene and genus specific primers

Target organisms	Amplicon size	Primer Sequence
mcrA gene-specific primer		
Methanogens (Luton et al., 2002)	470bp	F-5'-GTGGTGTMGGATTCACACARTAYGCWACAGC R-5'-TTCATTGCRTAGTTWGGRTAGTT
16S rRNA genus specific primers		
Methanospirillum (Rowe et al., 2008)	122bp	F-5'-AGTAACACGTGGACAATCTGCCCT R-5'-ACTCATCCTGAAGCGACGGATCTT
Methanoculleus (Franke et al., 2009)	262bp	F-5'-GGAGCAAGAGCCCGGAGT R'-5-CCAAGAGACTTAACAACCC
Methanosaeta (Rowe et al., 2008)	266bp	F-5'-GGGGTAGGGGTGAAATCTTGTAATCCT R-5'-CGGCGTTGAATCCAATTAAACCGCA
Methanosarcina (Franke et al., 2009)	325bp	F-5'-CCTATCAGGTAGTAGTGGGTGTAAT R-5'-CCCGGAGGACTGACCAAA

2.3   Results

2.3.1   Methanogenic activity

After drying and reconstitution in nutrient medium, MMC produced a significant amount

of CH_4 from H_2:CO_2 and calcium acetate (Figure 2.1 and 2.2). The relative methanogenic

activities of dried culture compared to non-dried culture were 65±5% for H_2:CO_2 and

41±10% for acetate after freeze drying, and were statistically different from each other.

The relative methanogenic activities after heat drying was 45±5% for H_2:CO_2 and 37±8%

for acetate assay and found to be statistically the same (Figure 2.2).
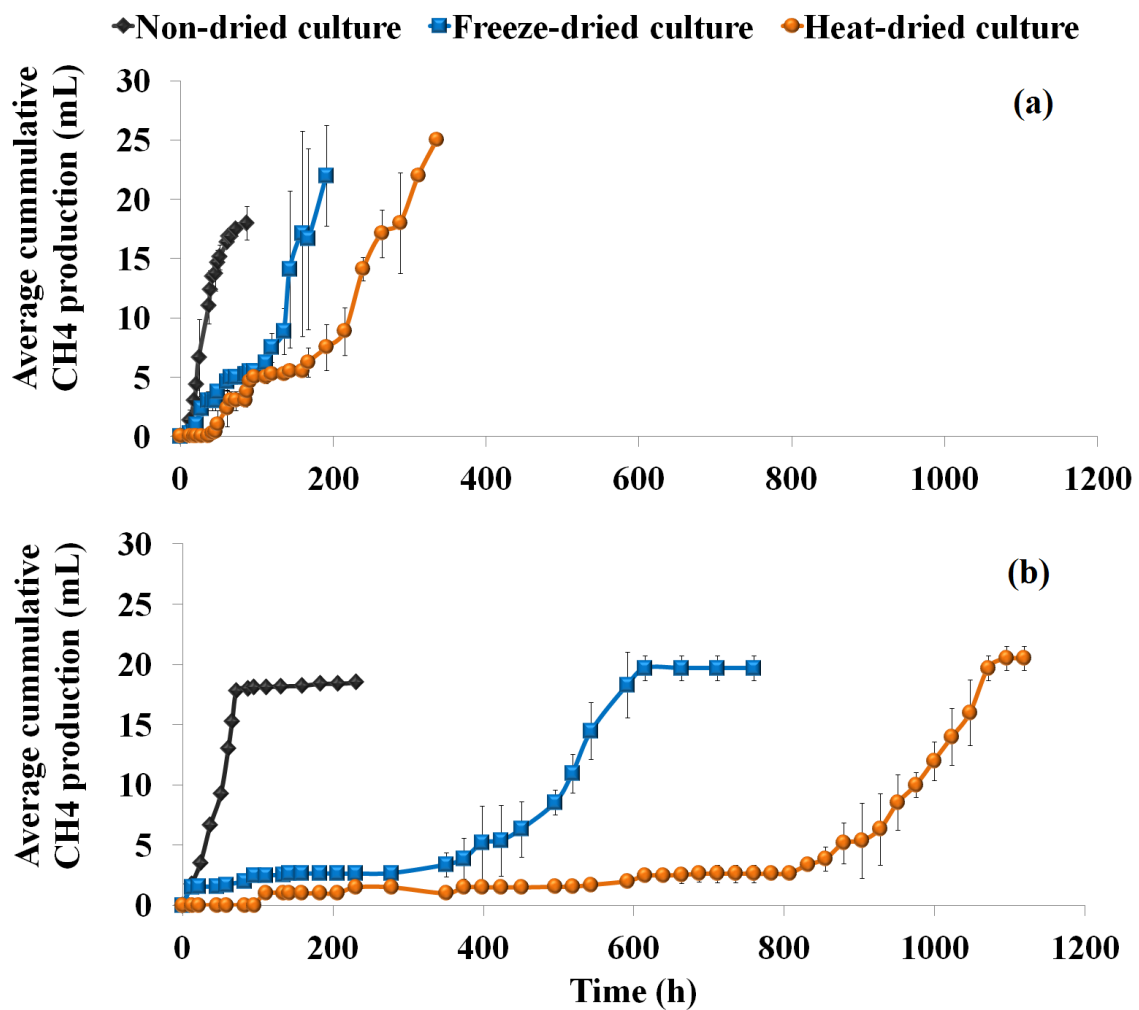
Figure 2.1. Average cumulative CH4 production rate of mixed methanogenic culture for (a) H$_2$:CO$_2$ (b) acetate assays before and after drying in air
Error bars represent the standard deviation of 4 replicates. Some error bars are small and not visible
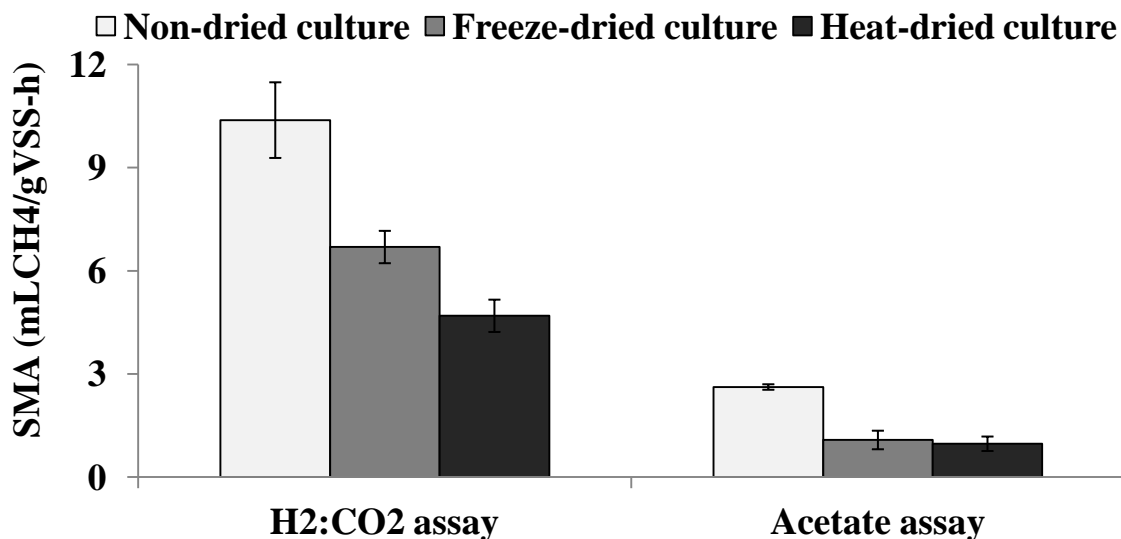
Figure 2.2. Specific methanogenic activity of mixed methanogenic culture before and after drying in air
Error bars represent the standard deviation of four replicates

The average activity lag was significantly higher for dried culture compared to the negligible lag observed for the non-dried culture (Figure 2.1). The average activity lag for non-dried culture was 21 hrs for $H_2:CO_2$ and 37 hrs for acetate assays. The average activity lag for freeze-dried culture was 152 hrs for $H_2:CO_2$ and 251 hrs for acetate assay whereas for heat-dried culture the average activity lag was increased to 450 hrs for $H_2:CO_2$ and 1000 hrs for the acetate assay. Furthermore, the relative average activity lag values for dried compared to non-dried cultures were significantly higher for acetate assays compared to $H_2:CO_2$ assays regardless of the drying method employed.

2.3.2 *mcrA* transcript and gene copies quantification

Metabolically active methanogenic populations in the MMC were analyzed by determining the number of *mcrA* transcript copies of non-dried and dried cultures after reconstitution of dried samples in nutrient medium (Figure 2.3). The relative *mcrA* transcripts copy value of dried culture compared to non-dried culture was 63±21% for

freze-dried culture, and 10±2% for the heat-dried culture respectively. Similarly, the relative *mcrA* gene copy value was 57±22% for the freeze-dried culture and 3±1% for heat-dried culture respectively. The correlation coefficient between *mcrA* gene and transcripts copy values was 99% (Figure 2.8, in Appendix I).
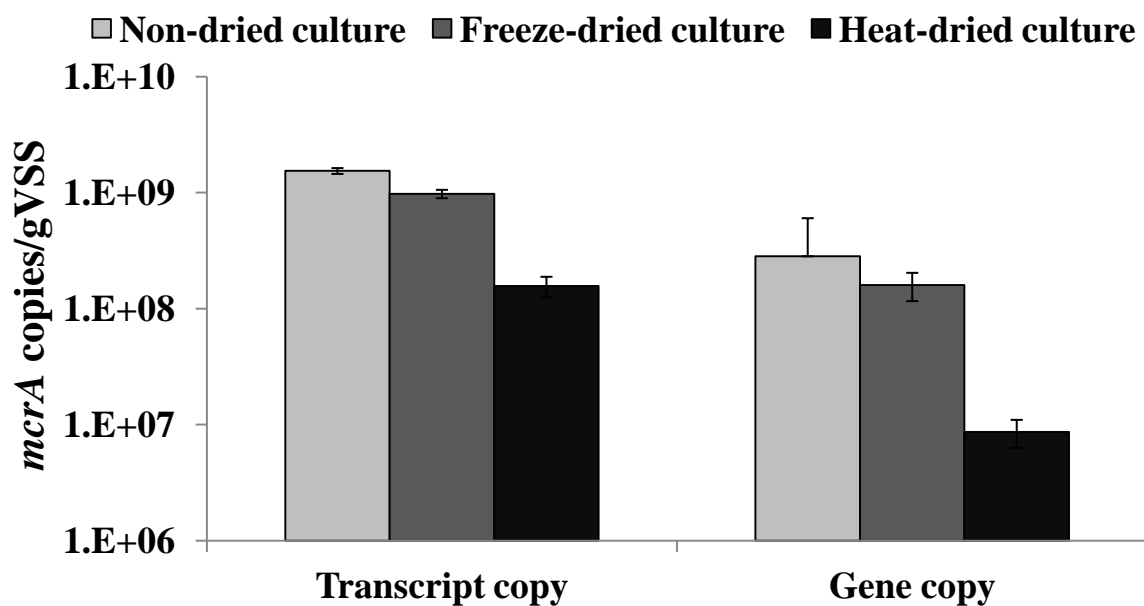
Figure 2.3. Quantification of *mcrA* transcript and gene copies of mixed methanogenic culture before and after drying in air
Error bars represent the standard deviation of six replicates

2.3.3   Relationship between SMA, activity lag and *mcrA* transcript copy numbers

The correlation coefficient between SMA values and *mcrA* transcript copy numbers was 70% for $H_2:CO_2$ assays and 92% for acetate assays. The correlation coefficients were 96% and 99% for $H_2:CO_2$ and acetate assay when *mcrA* transcript copy numbers correlated to average activity lag time (Figures 2.9 and 2.10, in Appendix I).

2.3.4   *mcrA* clone library

Analysis of the 72 clones included in the library of *mcrA* sequences provided a survey of the methanogen community present in the non-dried culture. The phylogenetic tree

demonstrated that the clones were closely related to three known genera:

Methanospirillum, Methanoculleus, and *Methanosaeta* (Figure 2.4).
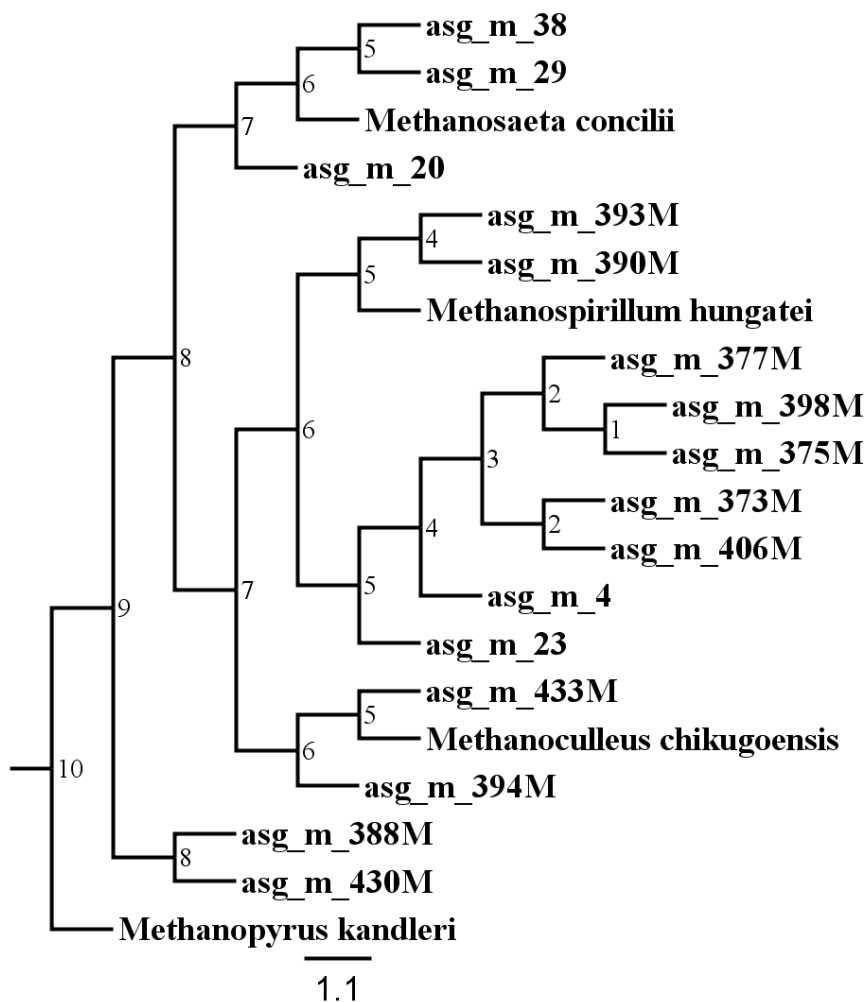
Figure 2.4. Phylogenetic tree of the non-dried mixed methanogenic culture

Calculations were based on the neighbor-joining algorithm (bootstrap number = 100). Numbers at node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Methanopyrus kandleri.* The clones were disposited in GenBank under the name "asg_m_".
This work was done by Dr. A.E. Schauer-Gimenez and Dr. R.L. Morris

The clone library was dominated by *Methanospirillum*-related sequences (85%), followed by sequences related to *Methanoculleus* (6%), and *Methanosaeta* (3%) (Figure 2.5). Seven percent of the *mcrA* sequences could not be assigned to a particular genus using 88% nucleotide sequence similarity as suggested by Steinberg and Regan (2008).

However, the rarefaction curve representing the number of unique clones leveled off after 55 clones were analyzed, indicating adequate coverage of the methanogenic community (Figure 2.6).
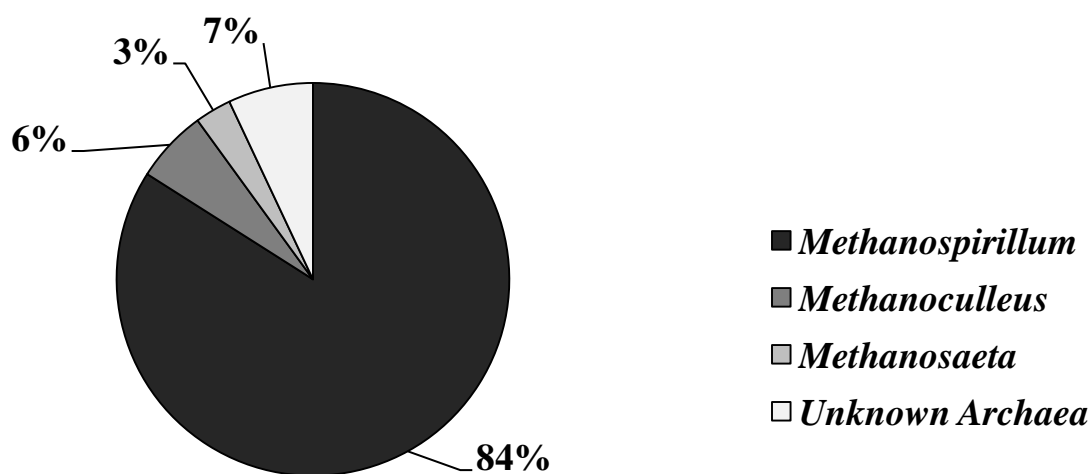
Figure 2.5. Abundence of methanogenic communities of non-dried mixed methanogenic culture based on *mcrA* gene
Clones were analyzed using restriction fragment length polymorphism (RFLP) and unique clones were sequenced. Clones were related at 88% similarities with GenBank® sequences.
This work was done by Dr. A.E. Schauer-Gimenez and Dr. R.L. Morris
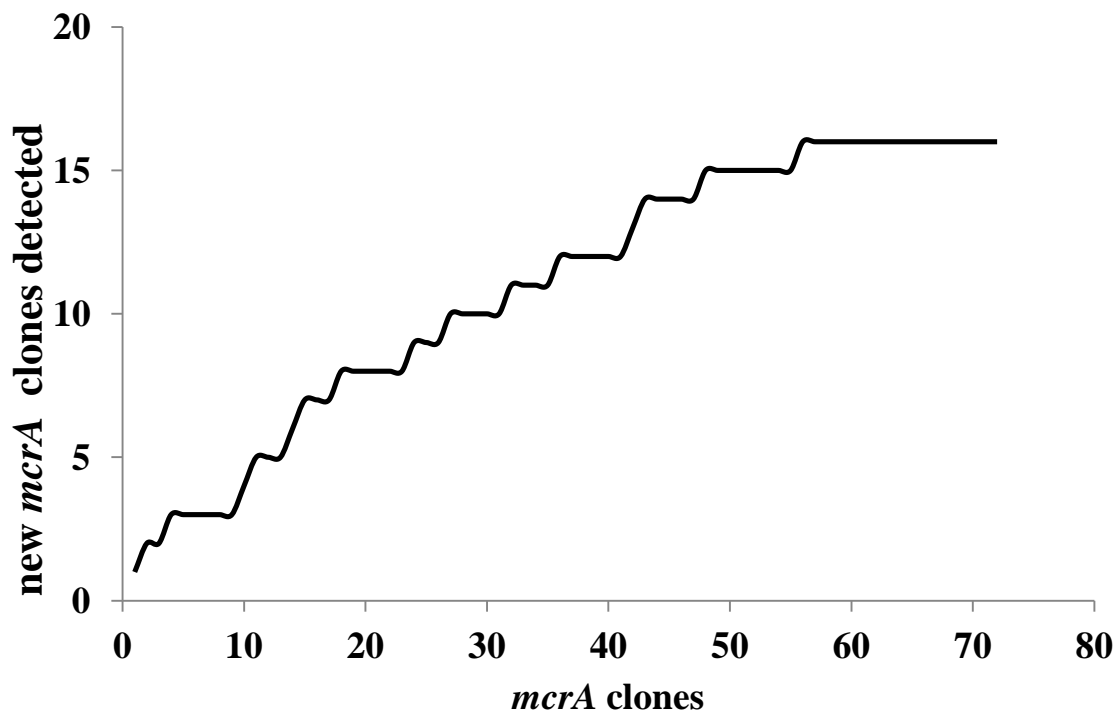
Figure 2.6. Rarefaction curve of *mcrA* clone library of non-dried mixed methanogenic culture
Total 72 new clones were analyzed
This work was done by Dr. A.E. Schauer-Gimenez and Dr. R.L. Morris

2.3.5   *16S rRNA* transcript and gene copy quantification

The *16S rRNA* transcript and gene copies were quantified for individual genera of

Methanospirillum, *Methanoculleus*, *Methanosaeta* and *Methanosarcina* using *16S rRNA*-

genus specific primers (Figure 2.7). The *16S rRNA* transcript copies estimated per gram

of VSS for *Methanospirillum*, *Methanosaeta*, and *Methanoculleus* were statistically

different among non-dried, freeze-dried and heat-dried cultures (Figure 2.7a). The

relative 16S rRNA transcript copy number of dried to non-dried culture was statistically

higher for *Methanospirillum*, followed by *Methanosaeta* and *Methanoculleus* for both

freeze-dried and heat-dried cultures, with 40±13%, 20±3% and 6±2% for freeze-dried

culture and 5±1%, 3±0.4% and 2±0.3% for heat-dried biomass, respectively. Similar results were obtained for *16S rRNA* gene copy numbers before and after freeze- and heat-drying (Figure 2.7b). The relative *16S rRNA* gene copy values for *Methanospirillum*, *Methanosaeta* and *Methanoculleus* were 51±11%, 28±14% and 4±3% for the freeze-dried culture and 4±1%, 0.6±0.3% and 0.6±0.6% for the heat-dried culture, respectively.

No *16S rRNA* transcripts or gene copies were detected for *Methanosarcina* for the non-dried and dried cultures. The correlation coefficient between *16S rRNA* transcript and gene copy values was 89% (Figure 2.11, in Appendix I).
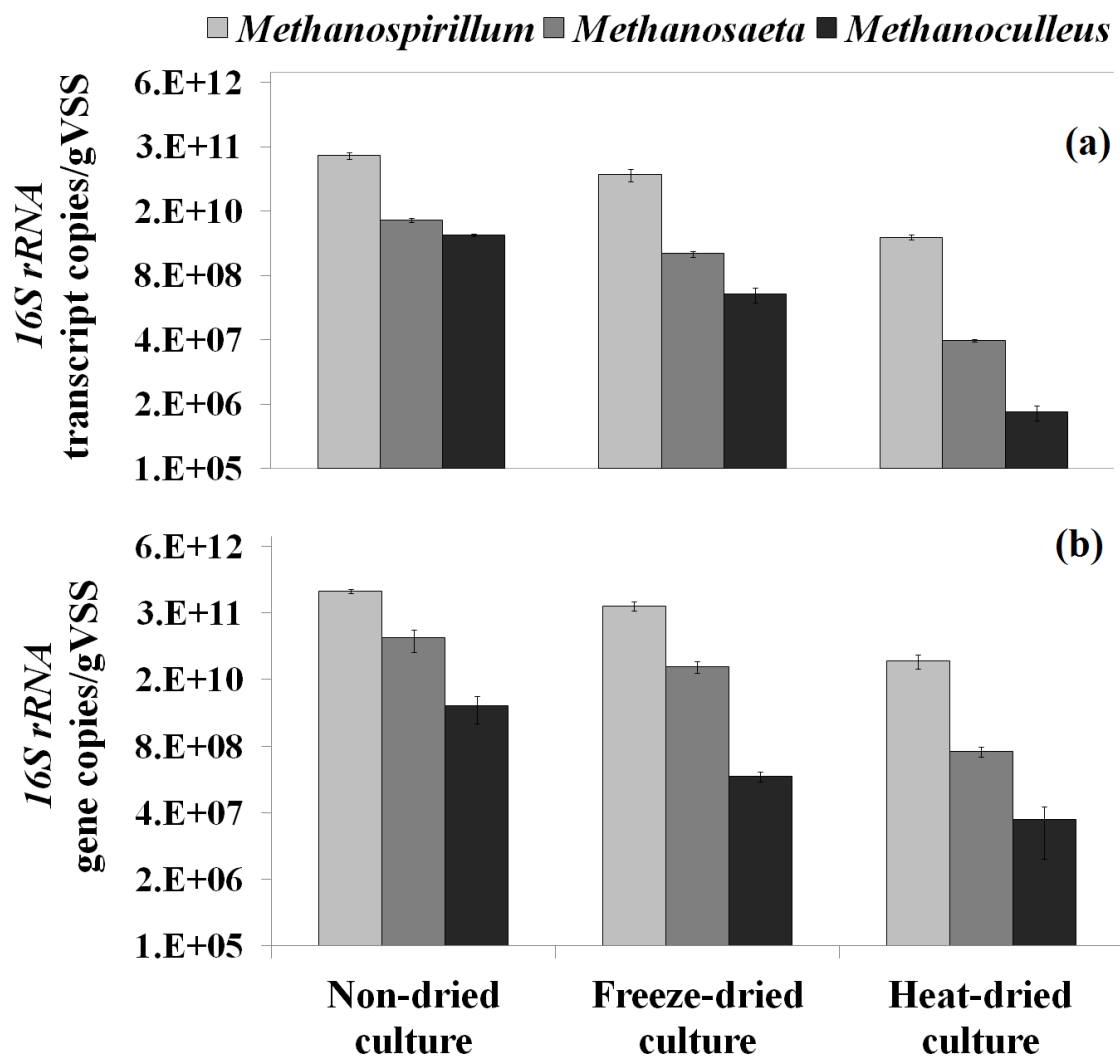
Figure 2.7. Quantification of *16S rRNA* copies of mixed methanogenic culture before and after drying in air
(a) Transcript copies and (b) Gene copies
Error bars represent the standard deviation of six replicates
The work was done collaborativly with Keerthi Cherukuri (Department of Biological Science, Marquette University)

2.4 Discussion

The methanogenic activities and qPCR results revealed significant functional activities of methanogenic populations even after drying under extreme temperature and in the presence of air. These results were not anticipated based on earlier literature that indicated the combined effect of drying under extreme temperature and air could be lethal to methanogens. Fetzer et al. (1993) and Liu et al. (2008) found a lethal effect of the combination of desiccation and O_2 exposure on pure cultures of methanogens. Five out of seven methanogenic strains lost their viability when liquid cultures were desiccated at ambient temperature in atmospheric air (Liu et al., 2008). Ueki et al. (1997) reported non-detectable methanogenic cell viability and no CH_4 production when methanogens in air-dried paddy soil were heated to 105 ^{0}C for 10 hrs. Our results contradict these previous findings and show significant methanogenic activity can be exerted, even after the culture exposed to heat (104 ^{0}C) drying for 12 hrs in the presence of air.

The reason for significant methanogenic population survival is speculated to be a result of some methanogens being inherently more tolerant to extreme conditions than previously estimated. Other researchers have discussed that cells do not die until functioning copies of critical components within cells decreases below a certain threshold levels (Miles, 2006; Nguyen et al., 2006). Lievense and van't Riet (1994) demonstrated that some microorganisms have increased heat resistance with decreasing water concentration. The denaturation temperature of protein in air dried *L. bulgarius* was reported to be as high as 200 ^{0}C (Oldenhof et al., 2005). In addition, some methanogens that survived air drying may have been shielded by surrounded non-methanogenic cells that developed as a physical barrier for direct O_2 diffusion. This possibly resulted in less damage being exerted to critical cell components and provided a better chance for cell

survival (Shen et al., 1996; Liu et al., 2008; Botheju and Bakke, 2011). The culture developed on non-fat dry milk as a substrate contained a high amount of carbohydrates and proteins. The residual concentration of these compounds in the culture suspension prior to drying may have acted as a protectant to drying stress. Several studies discussed the protective mechanism of carbohydrates, proteins and their derivatives including skim milk, resulting in protection of cellular structures and proteins from thermal and cryo-injuries exerted during drying (Leslie et al., 1995; Hubalek, 2003; Morgan et al., 2006; Santivarangkna et al., 2008). Some or all of these stated reasons may have increased the resistance of some methanogenic cells to tolerate severe stresses induced due to drying, high temperature and air exposure.

The overall negative impact of heat drying was significantly higher than freeze drying on methanogenic population survivability. The active methanogenic population estimated by *mcrA* transcript copy number was found to be significantly lower after heat drying than freeze drying (p=0.005). Similarly, the average relative activity lag was significantly greater after heat drying than freeze drying in both $H_2:CO_2$ (p=0.005) and calcium acetate SMA assays (p=0.005). The activity lag was considered to be the recovery time for the active cells to grow and attain a significant population. Also, methanogenic activity was lower after heat drying compared to freeze drying for the $H_2:CO_2$ assays (p=0.005).

High methanogenic cell inactivation after heat drying could be due to heat stress. Santivarangkna et al., (2008a) discussed that heat stress can severely damage critical components such as cellular structures, proteins, DNA, RNA and ribosomes and permanently damage cell functionality. Similarly, in freeze drying, freezing of cells at -

196 ^{0}C in liquid nitrogen leads to significant cryo-injury to critical cellular components

due to intracellular/extracellular ice formation and osmotic pressure imbalance within and

around the cell compared to subsequent drying (Simione and Brown, 1991; Morgan et al.,

2006; Santivarangkna et al., 2008). Furthermore, oxidative stress during drying

significantly increases cell damage (Franca et al., 2007). Decrease in the *mcrA* gene copy

values after drying indicated that the cell damage extended to the DNA molecules.

Although the estimated loss in *mcrA* gene copies could be a result of variation in DNA

extraction efficiencies of multiple samples, our results showed less than 10% variability

in extraction of duplicate samples (data not shown) which indicates that the DNA loss

was due to the drying process, and not an analytical artifact.

The *mcrA* clone library results revealed that the culture was a mixed

methanogenic community with three predominant methanogenic genera;

Methanospirillum, *Methanoculleus* and *Methanosaeta* (at 88% similarity). The members

of *Methanospirillum* and *Methanoculleus* are known to be hydrogenotrophic

methanogens which utilize H_2 as a main substrate to produce CH_4, whereas the members

of *Methanosaeta* are known to be acetoclastic methanogens which utilize acetate as a

substrate to produce CH_4. These methanogenic genera are commonly found in anaerobic

bioreactors treating municipal or industrial organic wastes and/or wastewaters (Leclerc et

al., 2004; Sawayama et al., 2005; Hori et al., 2006; Talbot et al., 2008; Lebuhn et al.,

2009; Goberna et al., 2010; Nelson et al., 2011). Other commonly reported methanogenic

genera in these systems include *Methanosarcina, Methanobacterium,*

Methanobrevibacter, and Methanolinea which were not detected in our culture (Leclerc

et al., 2001; McMohan et al, 2004; Rastogi et al., 2008; Steinberg and Regan. 2008; Goberna et al., 2010; McKeown et al., 2009).

The qPCR results using *16S rRNA* genus-specific primers indicated higher transcripts and gene copies of *Methanospirillum*, followed by *Methanosaeta,* and *Methanoculleus* and no copies were detected for *Methanosarcina.* These results were supported by *mcrA* clone library analysis, indicating that the relative values for different genera were reproducible, even when using different primers. The results followed a similar pattern after freeze- and heat-drying. Percent value of transcript and gene copies was higher for *Methanospirillum*, followed by *Methanosaeta,* and *Methanoculleus* for both, freeze- and heat-drying which suggested higher tolerance of *Methanospirillum* to drying than *Methanosaeta* genus. This corresponded to the observation of shorter relative activity lag time of dried culture in H_2:CO_2 activity assays compared to acetate assays. However, the *Methanoculleus* community was found to be sensitive to drying compared to other genus for higher damage or cell loss.

Variation in survival of *Methanospirillum*, *Methanosaeta, and Methanoculleus* may be explained by two phenomena: (1) differences in the culture's initial cell concentrations of each genus and (2) variation in the tolerance capability of each genus. Survival patterns of identified methanogenic communities after drying followed initial cell concentration before drying which indicate survival of methanogenic cells may be a function of initial cell concentration. Previous studies mentioned that the culture's initial cell concentration is a decisive factor for sufficient cell survival after drying (Costa et al., 2000; Miyamoto-Shinohara et al., 2000; Morgan et al., 2006). Secondly, genera may differ in their tolerance capability to exist in harsh environments due to their difference in

intrinsic defensive systems and extrinsic structures. Liu et al., (2008) reported different

tolerance capabilities of seven methanogenic strains. *Methanosarcina barkeri* was found

to be more aerotolerent than *Methanobrevibacter arboriphilus* or *Methanococcus voltae*

(Brioukhanov and Netrusov, 2007). Other authors reported greater tolerance of

Methanosarcina barkeri to desiccation and high pressure (1bar) or high temperature (100

^{0}C) desiccation compared to *Methanothermobacter wolfeii and Methanobacterium*

formicicum (Kendrick and Kral, 2006; Kral et al., 2011; Tryon et al., 2011).

Methanospirillum hungatei offered higher tolerance to natural desiccation during 3 to 14

days of air exposure, compared to *Methanosarcina mazei TMA* and no survivability for

Methanoculleus olentangyi (Liu et al., 2008). Morris (2011) identified higher survival of

Methanospirillum in microaerobic environment than *Methanoculleus.*

     Furthermore, the cell walls of *Methanospirillum* and *Methanosaeta* genera

possess thick, relatively impervious S-layers that form a so called "sheath" which

encompasses the cell walls. This ultra-structure has been reported to increase resistant to

heat and chemical treatment (Beveridge et al., 1993; Beveridge, 2002; Ma et al., 2006;

Claus and Konig, 2010). On the other hand, *Methanoculleus* is irregularly shaped, and its

S-layer structure is presumed to be less strong and easier to deform than others (Sprott

and Beveridge, 1993). Beveridge et al. (1993) reported a higher content of tetraether

lipids in the *Methanospirillum hungatei* GP1 membrane which provides stronger plasma

membrane to *M. hungatei* GP1 compared to *Methanosaeta concilii*. These variations in

cellular structure may result in different degrees of tolerence to cell damage during harsh

conditions. In addition, variation in cell surface area may provide varying resistance to

cell damage. Typically, higher cell surface area results in greater cellular structure damage (Carvalho et al., 2004).

2.5   Conclusion

Methanogens can survive the combined effect of drying under extreme temperature and air and after revival their functional activities return. Apparently, *Methanospirillum* is more tolerant/resilient to these conditions than *Methanosaeta*, and *Methanoculleus*. This unique tolerance ability of some methanogens challenged the conventional notion of methanogens sensitivity to O_2/air, extreme temperatures and drying. Methanogens can survive harsher conditions than previously considered, conceivably even an extreme environment such as that on Mars. In addition, heat and freeze-drying could be future approaches to preserve methanogens economically for commercial applications.

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2.7 Appendix I

Equation shown on chart:
$$y = 5132.7x^{0.65}$$
$$R^2 = 0.99$$
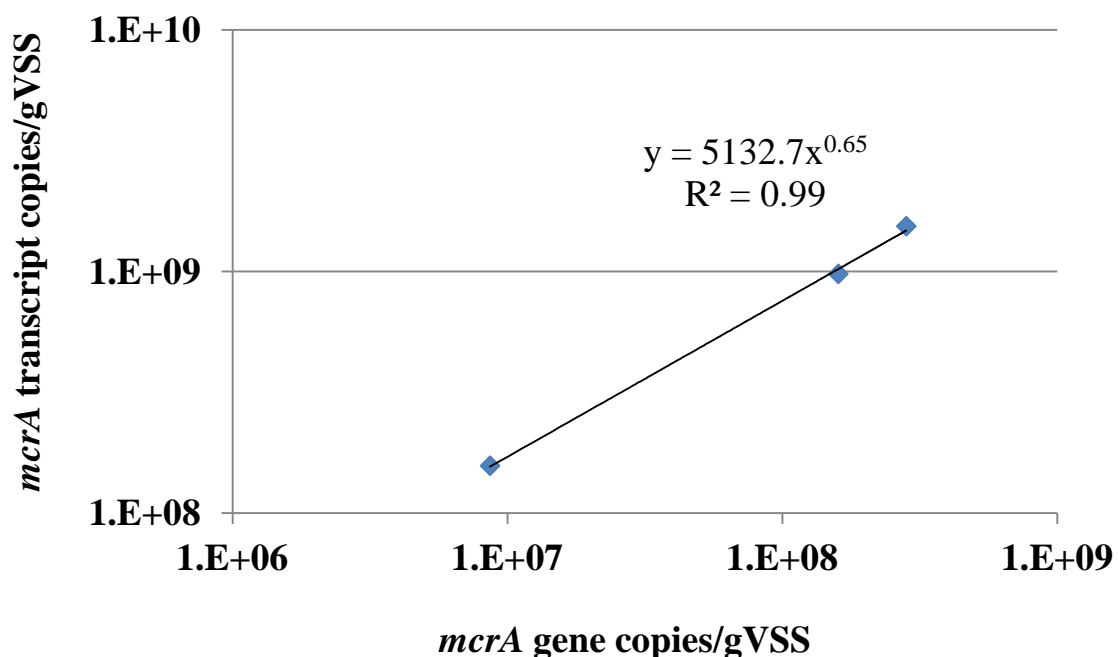
Figure 2.8. Relationship between *mcrA* transcript and gene copies of mixed methanogenic culture before and after drying
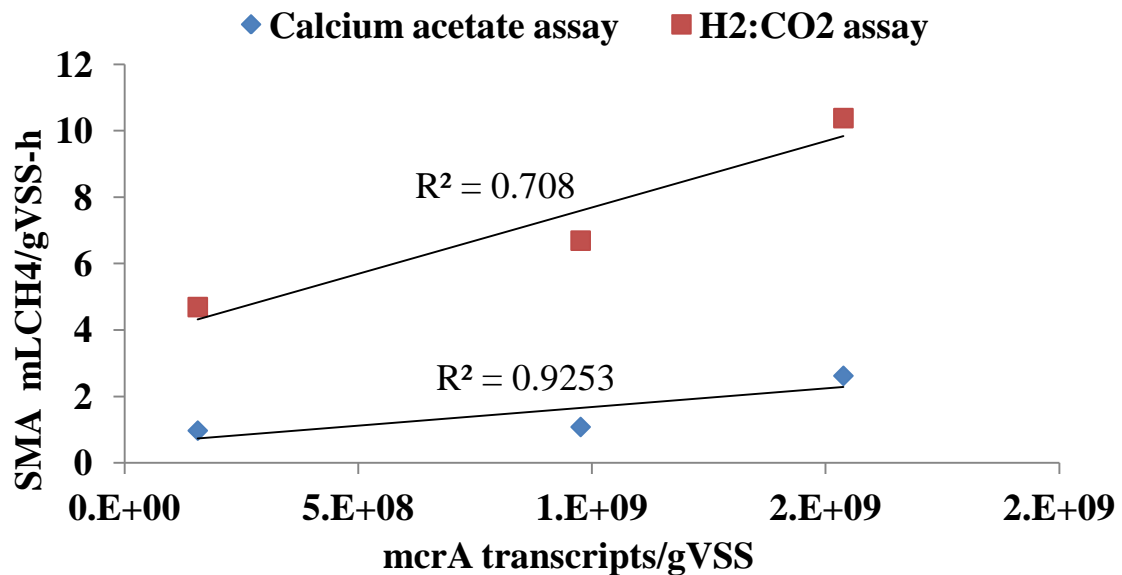
Figure 2.9. Ralationship between SMA and *mcrA* transcripts copies of mixed methanogenic culture before and after drying
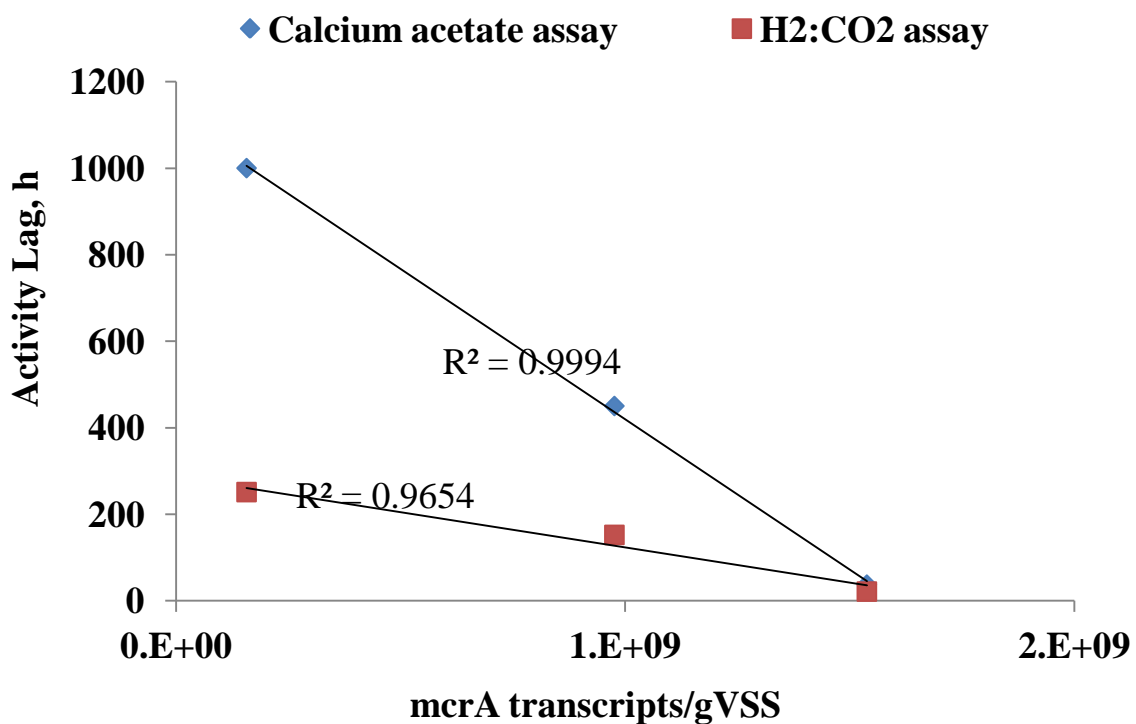
Figure 2.10. Ralationship between activity lag and *mcrA* transcripts copies of mixed methanogenic culture before and after drying
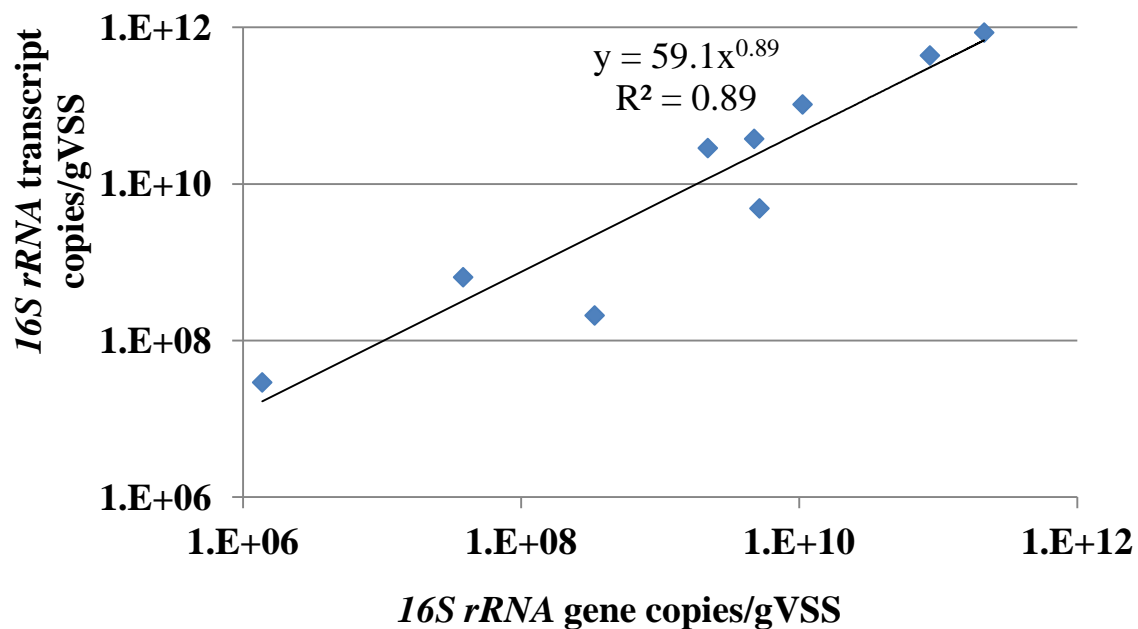
Figure 2. 11. Ralationship between *16S rRNA* transcripts and gene copies of mixed methanogenic culture before and after drying in air

Chapter 3 Methanogenic Culture Preservation and Long-Term Storage in Presence of Air

3.1   Introduction

The use of anaerobic biotechnology is increasing as a sustainable process to treat high-and low-strength organic wastewaters and produce CH_4 as a fuel (McCarty et al., 2011). CH_4 is an end product in anaerobic processes, produced biologically by a methanogenic community having syntrophic relationships among different trophic groups (Speece, 2008). Methanogens play the key role of maintaining a low H_2 concentration to thermodynamically drive unfavorable biochemical fermentation reactions and keep the digestion process stable and efficient (McCarty and Smith, 1986). However, relying on undefined microbial communities that result from the current engineering practice of seeding a digester with any available biomass may not be an effective approach to optimize the anaerobic process. Emerging molecular tools are now being used to understand the relationship between various microbial community structures and digester function (Morris, 2011; Tale et al., 2011). The progressive understanding of anaerobic microbial communities may reveal benificial microorganisms to improve process stability and CH_4 production which may lead to the production of a number of customized methanogenic microbial communities for different anaerobic applications.

In general, growth of methanogenic communities takes a longer time compared to growth of fermentative bacterial communities due to slow growth kinetics of methanogens (Ribes et al., 2004). Therefore, easy preservation of various defined methanogenic cultures would be beneficial for research and practical applications. Customized methanogenic cultures could be preserved and used for future research, standardized testing, seeding of laboratory and full-scale reactors, and bioaugmentation

of existing digesters to improve performance. Recent research revealed that culture selection to seed anaerobic bioreactors, based on methanogenic activity, can reduce reactor start-up time and improve steady-state performance (Tale et al., 2011). Others showed that bioaugmentation using enriched methanogenic cultures can be a beneficial approach to improve increase the degradation rate of difficult-to-degrade halogenated and aromatic organic compounds (Hajji et al., 2000; Da Silva 2004), reduce bioreactor start-up time (Guiot et al., 2000), increased CH_4 production in pschycrophillic bioreactor (Qiu et al., 2011), and shortened reactor recovery time after organic overload (Tale et al., 2011) or exposure to a toxicant (Schauer-Gimenez et al., 2010).

Common practices to preserve microbial cultures are freezing, refrigeration and drying (Castro et al., 2002). Preservation and subsequent storage of microbial cultures by freezing or refrigeration are often impractical on a commercial scale due to handling of high volumes, culture instability during storage, and energy intensive storage at lower temperatures. Preservation of microbial cultures by drying reduces the mass, facilitates less expensive shipping and handling and can provide a relatively stable product during storage (Aguilera and Karel, 1997; Santivarangkna et al., 2007). In bio-industries, spray drying and freeze drying are the most common methods to preserve a large mass of microbial culture (To and Etzel, 1997; Wang et al., 2004; Morgan et al., 2006). Spray drying is a high-temperature, high-throughput process that results in rapid, less expensive drying (Santivarangkna et al., 2007; Peighambardoust et al., 2011). Spray drying is widely used to preserve lactic acid bacteria at an outlet product temperature of 80 to 105 ^{0}C (Gardiner et al., 2000; Desmond et al., 2002; Corcoran et al., 2004). On the other hand, freeze drying is an ultra-low temperature drying process requiring long drying time

and involves high capital and energy costs due to the low operating temperature and pressure (Claussen et al., 2007; Santivarangkna et al., 2007). However, freeze drying is a well-accepted technique to preserve a variety of heat- and O_2-sensitive microorganisms (Staab and Ely, 1987; Malik, 1990; Miyamoto-Shinohana et al., 2000; Cleland et al., 2004; Morgan et al., 2006; Miyamoto-Shinohana et al., 2008).

Very limited literature is available on preservation of methanogenic cultures using drying. This is probably due to the perceived sensitivity of methanogens to drying and O_2 toxicity. Drying exerts severe stress on microbial cells by removing the water molecules associated with cellular components. This can lead to significant damage to the cell membrane and cellular proteins which causes a significant loss in cell viability (Teixeira et al., 1995; Potts, 2001; Santivarangkna et al., 2008a). Previous studies have shown that sugar additives can reduce the cell drying stress mainly due to replacement of cellular water by sugar hydroxyl groups and the ability of sugars to vitrify forming glassy amorphous solids around the cell (Leslie et al., 1995; Conrad et al., 2000; Carvalho et al., 2004; Morgan et al., 2006; Hubalek, 2003; Santivarangkna et al., 2008). This phenomenon provides higher stability of cell components during drying and subsequent storage and preserves greater cell viability. Methanogens are strict anaerobes and non-spore forming microorganisms. Oxidative stress caused by reactive O_2 species (ROS) during preservation and storage can severely damage the cell membrane, DNA, proteins and specific functional enzymes causing cell death (Schonheit et al., 1981; Brioukhanov and Netrusov, 2007; Liu et al., 2008). Therefore, methanogenic culture preservation and subsequent storage is typically considered to require anaerobic conditions (Castro et al., 2002; Cleland et al., 2004; Iino and Suzuki, 2006) which can make the process

cumbersome and expensive. However, previous studies have revealed the limited tolerance of methanogens to oxic conditions (Zitomer and Shourt, 1998; Angel at al., 2011; Angel at al., 2012; Botheju and Bakke 2011). Furthermore, our previous study demonstrated tolerance of some methanogens to drying under extreme temperatures in the presence of air.

The present study was performed to determine the survivability of enriched hydrogen-utilizing methanogenic cultures and the preservation of activity after long-term storage in the presence of air, as well as the effectiveness of glucose as a protectant.

3.2   Methods

3.2.1   Culture development

Four enriched hydrogen-utilizing methanogenic cultures (HMCs) were developed. Recently, Schauer-Gimenez et al. (2010) successfully demonstrated improved recovery of transiently upset anaerobic digesters by bioaugmenting them with the HMC biomass used in this study. Development of the enriched HMC was described by Schauer-Gimenez et al. (2010). In brief, 2 L laboratory bioreactors were seeded with mesophillic biomass from an anaerobic digester at a municipal wastewater treatment plant. Reactors were operated in daily feed-and-draw mode over 2 years at 35 ^{0}C and a 15-day retention time. All HMCs (1, 2, 3 and 4) received basal nutrient medium (Schauer-Gimenez et al., 2010), and H_2:CO_2 (1:1, v/v ratio) in the headspace daily. In addition, HMC 2 received glucose (40 mg/L-day), whereas HMC 3 received O_2 in the form of air (80 mg O_2/L-day), and HMC 4 received both glucose and O_2. The dose of O_2 satisfied less than 6% of the theoretical oxygen demand.

3.2.2 Preservation methods

Biomass was collected from all HMC bioreactors over 3 days and kept at 4 ^{0}C in glass

bottles sparged with N_2:CO_2 gas (7:3 v/v) to obtain a sufficient volume (0.3 L) for

preservation. Biomass was then preserved and subsequently stored in both wet and dried

forms. Drying was accomplished using either freeze- or heat-drying. Biomass with

volatile suspended solids (VSS) concentrations of 200 to 500 mg/L was centrifuged (2.5

gravitational force, 4500 rpm) for 10 min to thicken the biomass to 3 to 7.5 g VSS/L

before drying or concentrated to12 to 30 g VSS/L before wet storage. Prior to drying or

wet storage, some centrifuged biomass samples were amended with 20% glucose, (1:1

v/v) as a protectant (Colleran et al., 1992; Hubalek, 2003; Strasser et al., 2009), whereas

other biomass samples were not amended with glucose.

For heat drying, 20 mL of centrifuged biomass was transferred to a 25-mL

porcelain crucible and dried in an oven at 104 ^{0}C for 12 hrs in the presence of air (Oven

1350GM, Sheldon Manufacturing Inc., Cornelius, OR, USA). For freeze drying, 20 mL

of centrifuged biomass was shell frozen by swirling a freeze-drying flask in liquid

nitrogen (Staab and Ely, 1987) for 7 min in the presence of air. Care was taken to create a

thin layer of biomass by spreading it inside the freeze-drying flask surface. Frozen

biomass was then dried using a bench-top freeze dryer (3GenMP Opti-Dry, Millrock

Technology, Kingston, NY, USA) at a condenser temperature of -45 ^{0}C and 13.3 Pa

pressure (Staab and Ely, 1987; Porubcan, 1991; Simione and Brown, 1991). The primary

and secondary drying times were determined by monitoring flask temperature over time

using a thermocouple (800024, Sper Scientific Ltd., Scottsdale, AZ, USA). Primary

drying was assumed to be complete when the flask temperature increased to the ambient

value. Secondary drying was performed for a period of time equal to that for primary

drying as recommended by others (Porubcan, 1991).

3.2.3 Short-term and simulated long-term storage

Residual moisture content (RMC) of dried and centrifuged wet biomass was quantified

by measuring total solids (TS) mass by standard methods (APHA et al., 2005). Dried

biomass was temporarily stored in a desiccator containing a $CaSO_4$ dessicant (Drierite[®],

W. A. Hammond Co., Xenia, OH, USA) at room temperature (20 to 23 ^{0}C) for 2 days

before activity testing. Long-term storage was simulated by holding dried biomass in a

desiccator at an elevated temperature of 35^{0}C for 15 days. This has been shown by others

to simulate storage for 20 years at 5°C under vacuum for many microorganisms (Sakane

and Kuroshima, 1997). During storage, dried biomass was exposed to air in the desiccator

headspace. Centrifuged wet biomass (≤5 mL volume) was stored in a sealed, 50-mL

centrifuge tube containing air in the headspace at room temperature for up to 15 days.

3.2.4 Actual Storage over five months

To measure viability over storage time, cultures 3 and 4 were heat dried as described

above and transferred to glass chromatography vials (30 mL) closed with taflon coated

rubber septa and plastic closures. The vials were stored in a desiccator at 4 ^{0}C.

(Miyamoto-Shinohana et al., 2000; Castro et al., 2002;Desmond et al., 2002; Morgan et

al., 2006; Miyamoto-Shinohana et al., 2008).   Methanogenic activity was measured

periodically for up to five months to determine the activity change during storage.

3.2.5 Methanogenic activity testing

Specific methanogenic activity (SMA) was measured as the maximum CH_4 production

rate per gram of biomass and was expressed as mL CH_4/g VSS-h. SMA testing was

performed using both H_2:CO_2 and acetate as substrates. Activity lag was also measured as the time required to obtain the maximum CH_4 production rate.

3.2.5.1   SMA for H_2

The SMA for H_2 was determined using the protocol described by Coates et al. (1996). Dried biomass was rehydrated in 300 mL of nutrient medium containing L-cysteine hydrochloride (500 mg/L) as a reducing agent (Coates et al., 1996). Serum bottles (160 mL) were charged with 25 mL of biomass suspension ($\leq$ 300 mg VSS/L). The bottle headspace was sparged with a N_2:CO_2 gas mix (7:3 v/v) for one minute and sealed with black balch-type rubber septa and aluminum seals. Bottles were incubated at 35^0C and 150 rpm in an incubator-shaker (C25KC, New Brunswick Scientific, Edison, NJ, USA) for 48 hrs and the biogas produced due to residual COD was released. Test bottles were then pressurized by injecting 100 mL of the H_2:CO_2 gas mix (4:1 v/v). The volume of gas remaining was measured over time using a glass syringe with a water-lubricated glass plunger, and subsequently re-injected into the serum bottle.

The H_2:CO_2 gas utilized at a given time was determined as the difference in the gas volumes (initial gas volume minus volume remaining) plus the gas volume produced from endogenous control bottles. CH_4 production was estimated as the volume of H_2:CO_2 gas utilized divided by the stoichiometric ratio 4 (i.e., 4 moles of H_2 and 1 mole of CO_2 consumed per 1 mole of CH_4 produced). Maximum CH_4 production rate (mL CH_4/h) was calculated by linear regression using the steepest slope for a minimum of three points on a graph of cumulative CH_4 produced versus time. SMA was determined by dividing the maximum CH_4 production rate by the VSS mass. SMA tests, endogenous controls, and

abiotic controls were run in triplicate. Gas production in endogenous controls and gas leakage in abiotic controls were negligible.

3.2.5.2 SMA for acetate

The SMA for acetate was determined using the protocol described by Anagelidaki et al. (2007). Serum bottles (160 mL) were charged with 25 mL of biomass suspension (< 2 g/L VSS) and sparged with a $N_2:CO_2$ gas mix (7:3 v/v). After 48 hrs of incubation at 35 ^{0}C and removing the gas produced from residual COD from seed biomass, calcium acetate at 10 g/L was added. The biogas produced was measured over time with the gas displacement method using a glass syringe. The CH_4 production was estimated as the CH_4 volume increase less the endogenous control bottle CH_4 volume. Maximum CH_4 production rate (mL CH_4/h) and SMA (mL CH_4/g of VSS-h) were calculated as described in the SMA test for H_2.

3.2.6 Analysis

Data were analyzed using SPSS statistical program (Statistic Base 17.0). An analysis of variance was performed on the data and Tuckey's honestly significant difference (HSD) statistic was used for multiple comparisons of means at the 5% significance level.

1) Relative methanogenic activity after preservation or after preservation and storage (%)

$$= \frac{\text{SMA2 or SMA3}}{\text{SMA1}} \times 100 \ldots \ldots \text{Eqn 1.}$$

2) Rate of relative methanogenic activity loss of preserved culture during 15 days of simulated long-term storage (%)/day

$$= \frac{\left(\frac{\text{SMA2} - \text{SMA3}}{\text{SMA2}} \times 100\right)}{15} \ldots \text{Eqn 2.}$$

Where,

SMA1: Methanogenic acitivity before preservation, mLCH$_4$/gVSS-h

SMA2: Methanogenic activity after preservation, mLCH$_4$/gVSS-h

SMA3: Methanogenic activity after preservation and simulated long-term storage,

    mLCH$_4$/gVSS-h

Average methanogenic activity was an average of methanogenic activities of all HMC

cultures (1, 2, 3 and 4).

3.3 Results

All HMCs exhibited methanogenic activity before preservation as well as after

preservation and simulated long-term storage even though all were exposed to air during

drying, handling and storage (Table 3.1, Figure 3.1). The methanogenic activity of HMCs

before preservation was found to be very low for acetate; therefore further acetate SMA

analysis was not performed. The results discussed in this section are based on

methanogenic activity measured for H$_2$. The methanogenic activities of cultures 1, 2, 3,

and 4 before preservation were 216 ±30, 66 ±16, 240 ±22, and 60 ±8.5 mLCH$_4$/gVSS-h,

respectively, over the entire test period. The SMA values of cultures 1 and 3 that did not

receive glucose were significantly higher than those of cultures 2 and 4 that did. Similar

patterns were observed after preservation and simulated long-term storage. Average

methanogenic activities for each culture before preservation were not statistically

different over the entire study period, indicating overall quasi steady-state performance of

the bioreactors.

Table 3.1. Methanogenic activity of hydrogen-utilizing methanogenic cultures before and after preservation and after simulated long-term storage

	Methanogenic activity values (mLCH$_4$/gVSS-h)											
Preservation method	Before preservation				After preservation				After preservation and simulated long-term storage			
	HMC1	HMC2	HMC3	HMC4	HMC1	HMC2	HMC3	HMC4	HMC1	HMC2	HMC3	HMC4
With glucose addition												
Heat drying	275±22	61±6	271±9	58±2	154±8	40±5	110±27	38±2	119±10	20±2	108±12	21±2
Freeze drying	209±3	84±3	261±8	70±2	163±3	61±5	228±10	51±1	101±0	31±2	172±7	36±8
Centrifuging	187±0	43±1	207±6	54±0	135±0	34±6	224±3	44±3	19±8	9±3	27±9	15±6
Without glucose addition												
Heat drying	201±2	76±0	231±4	47±1	67±21	40±0	123±13	30±2	48±12	24±1	97±9	17±1
Freeze drying	204±0	83±0	230±1	73±1	144±18	48±0	168±2	45±2	53±8	16±5	110±4	23±3
Centrifuging	220±0	51±0	242±4	67±1	177±4	37±3	220±3	57±5	48±8	10±7	87±6	20±5

Table 3.2. Percent residual moisture content (RMC) of hydrogen-utilizing methanogenic cultures after preservation

Preservation method	HMC 1	HMC 2	HMC 3	HMC 4
With glucose addition				
Heat drying	2.1	1.6	2.2	3.9
Freeze drying	12	11	8.9	12
Centrifuging	90.2	91.2	89.3	91.5
Without glucose addition				
Heat drying	1.6	2.1	1.7	3.2
Freeze drying	10.5	11	9	12
Centrifuging	91.5	90.1	93.3	90.1
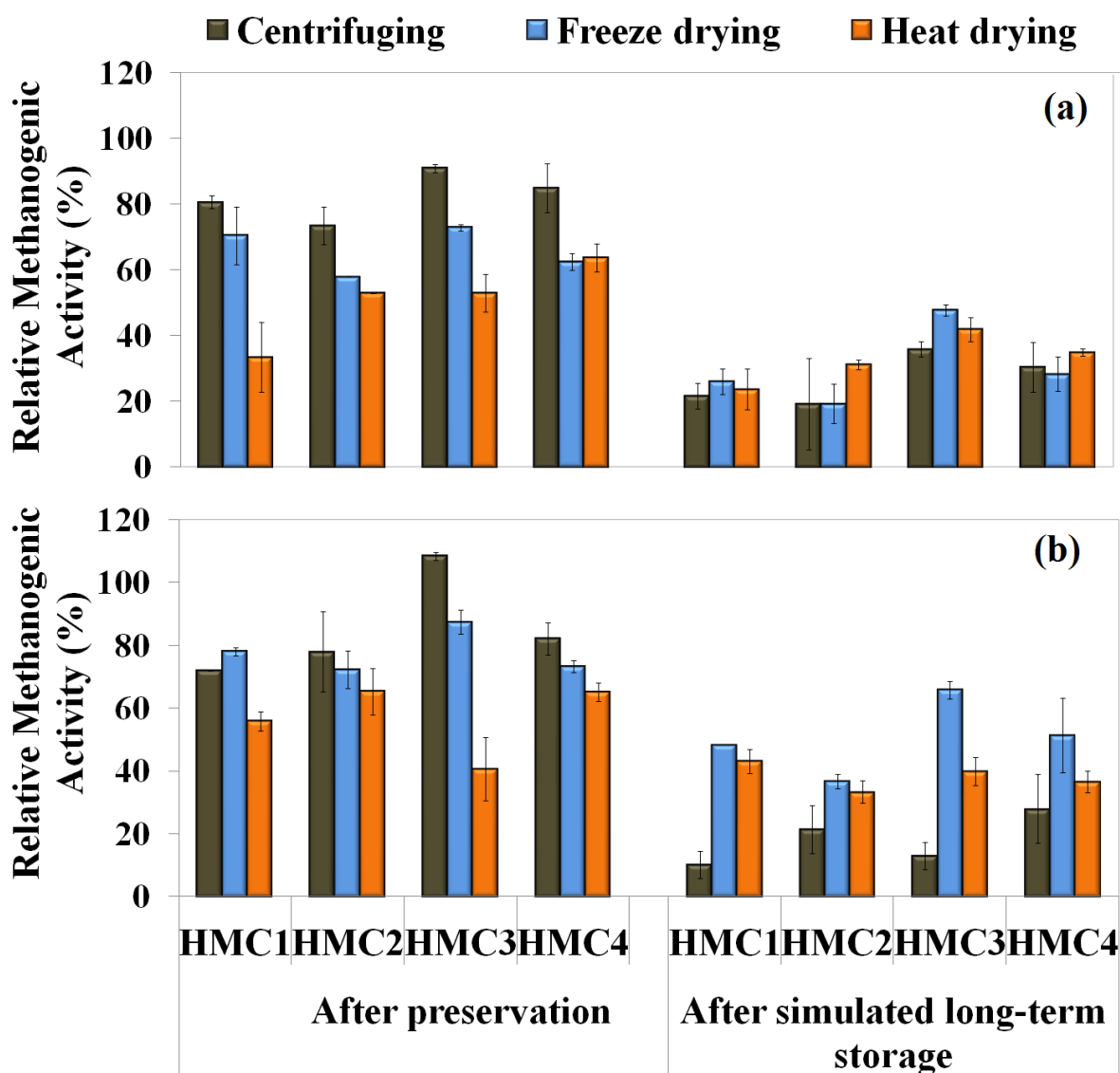
Figure 3.1. Relative methanogenic activity of hydrogen-utilizing methanogenic cultures after preservation, and after simulated long-term storage
(a) Without glucose addition, (b) With glucose addition
Error bars represent the standard deviation of three replicates. Some error bars are small and not visible

3.3.1   RMC and drying time of dried cultures

The culture's RMC after preservation is presented in Table 3.2. The average RMC for all

preserved cultures was 11 ±1.5% after freeze drying, 2.2 ±1% after heat drying 91±2 %

after biomass centrifuging (Table 3.2). In freeze-drying, primary drying was reached when ambient temperature was achieved after 12 hrs. Secondary drying was then continued to remove bound water for an additional 12 hrs and the freeze drying was completed after 24 hrs (Figure 3.8, in Appendix II).

3.3.2 Methanogenic activity without glucose addition

After preservation the average relative methanogenic activities estimated were 82 ±8% for centrifuged cultures, 66 ±7% for freeze-dried cultures and 51 ±13% for heat-dried cultures, respectively (Figure 3.2a). After preservation and simulated long-term storage, the average relative methanogenic activities observed were 27 ±10% for centrifuged cultures, 31 ±12% for freeze-dried cultures and 33 ±3% for heat dried cultures, respectively, and the values were not statistically different from each other.

The rate of relative methanogenic activity loss of preserved cultures was statistically different under the different preservation methods. The average relative methanogenic activity loss for all preserved cultures without glucose addition was 4.5 ±0.6 %, 3.5 ±0.9% and 2.2 ±0.4 % per day, over 15 days of simulated long-term storage for centrifuged, freeze-dried, and heat-dried cultures, respectively.

Figure 3.2. Average relative methanogenic activity of all hydrogen-utilizing methanogenic cultures after preservation, and after simulated long-term storage
(a) Without glucose addition, (b) With glucose addition
Error bars represent the standard deviation of twelve replicates. Some error bars are small and not visible
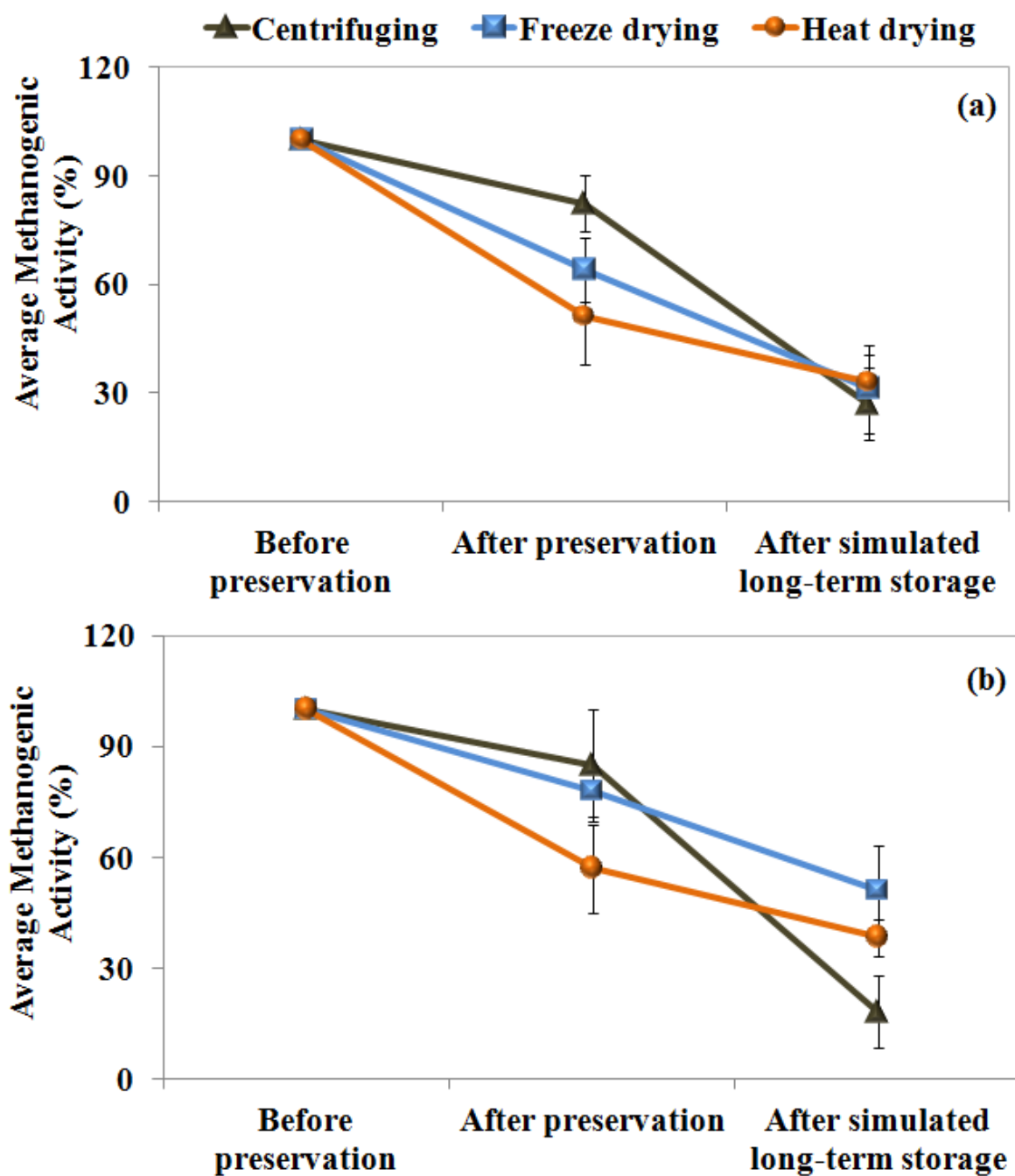
3.3.3 Methanogenic activity with glucose addition

After preservation, the average relative methanogenic activity was 85 ±16% for

centrifuged cultures, 78 ±7% for freeze-dried cultures and 57 ±12% for heat-dried

cultures, respectively (Figure 3.2b). Glucose addition resulted in a 17 % increase in

average relative methanogenic activity after freeze drying. In heat drying and biomass

centrifuging, the methanogenic activity was not statistically different from that observed

during non-glucose addition tests.

After preservation and simulated long-term storage, the average relative

methanogenic activities preserved was 18 ±10% for centrifuged cultures, 51 ±12% for

freeze-dried cultures and 38 ±5% for heat-dried cultures, respectively, which were

statistically different from each other. The average relative methanogenic activity

preserved after simulated long-term storage with glucose was 38% higher for freeze-dried

cultures and 16% for heat-dried cultures compared to the non-glucose tests. However,

glucose addition exerted a negative effect on centrifuged cultures.

3.3.4 Activity lag

A significant lag was observed in the methanogenic activity of preserved cultures

compared to the negligible lag in non-preserved cultures during SMA testing (Figure 3.3

and 3.4).
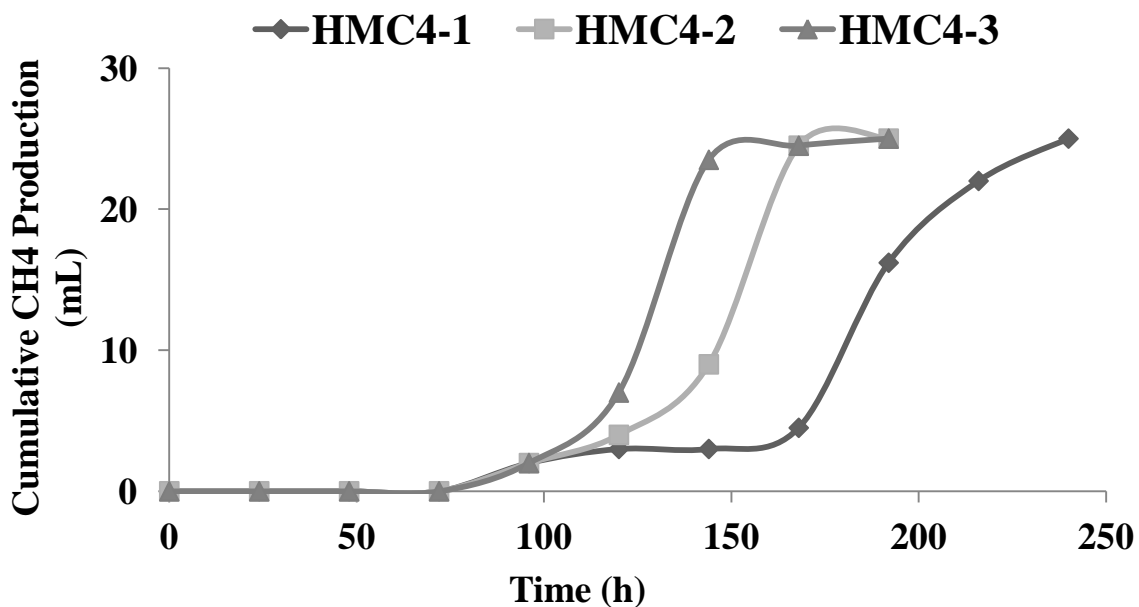
Figure 3.3. Methane production of heat-dried hydrogen-utilizing methanogenic cultures
Number 1, 2, and 3 are the replicates of HMC 4
This figure is used as an example to represent the activity lag and maximum CH_4 production rate of the preserved cultures

In non-glucose addition tests, the average lag value of all the cultures was statistically different for all preservation methods (Figure 3.4a). After preservation, the average activity lag value was greater for heat-dried cultures than freeze-dried and centrifuged cultures. After preservation and simulated long-term storage, the average activity lag value was significantly higher for centrifuged cultures compared to freeze- and heat-dried cultures.

In glucose added tests, the activity lag values were statistically lower for freeze-dried and heat-dried cultures compared to non-glucose added tests (Figure 3.4b). However, no statistical difference was observed in average lag values of centrifuged cultures with or without glucose addition.
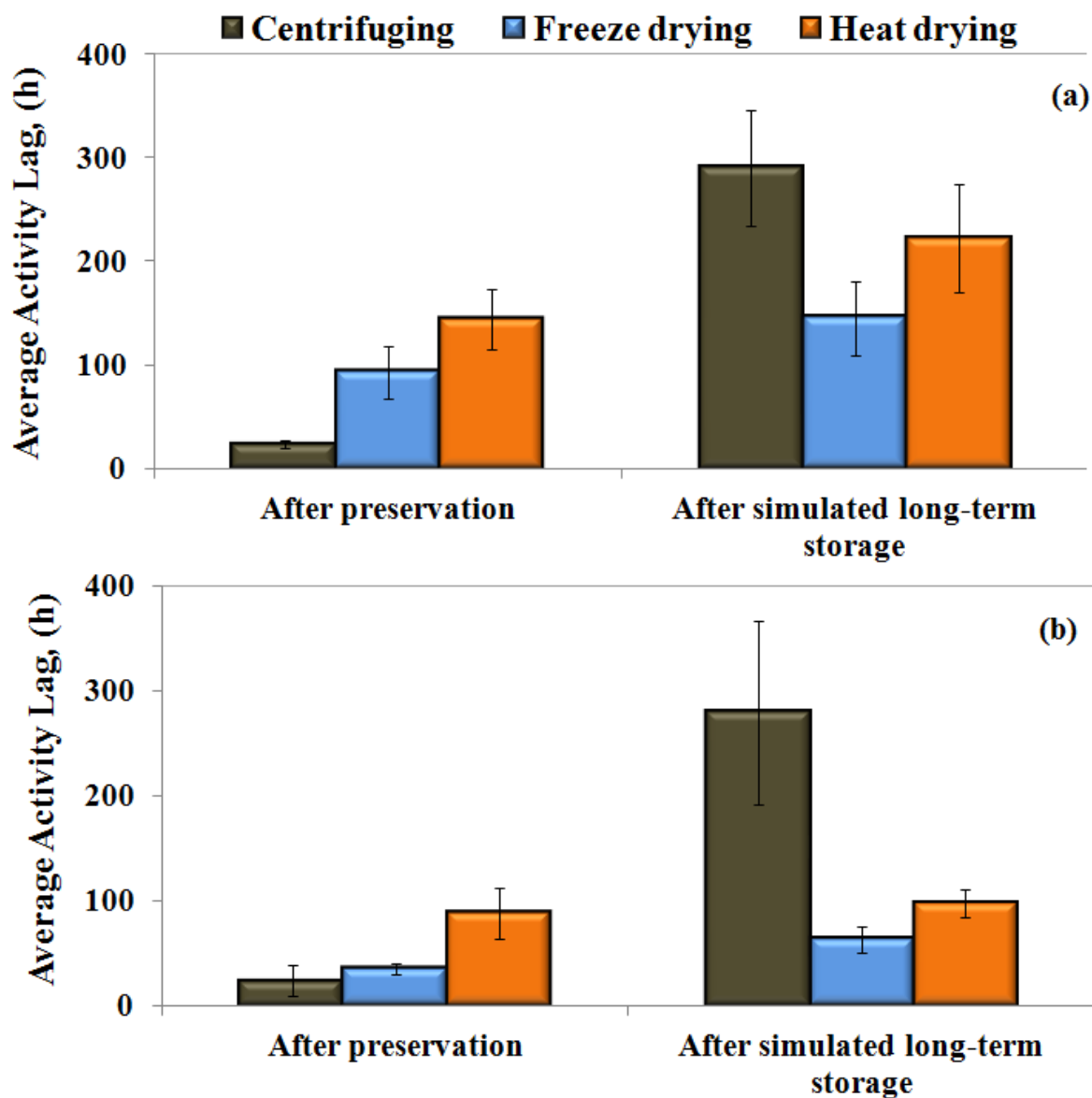
Figure 3.4. Average activity lag of all hydrogen-utilizing methanogenic cultures after preservation, and after simulated long-term storage (a) Without glucose addition, (b) With glucose addition
Error bars represent the standard deviation of twelve replicates. Some error bars are small and not visible

The activity lag was inversely proportional to methanogenic activity. Figure 3.5 showed the linear relationship between activity lag and relative methanogenic activity of preserved cultures with and without glucose addition.
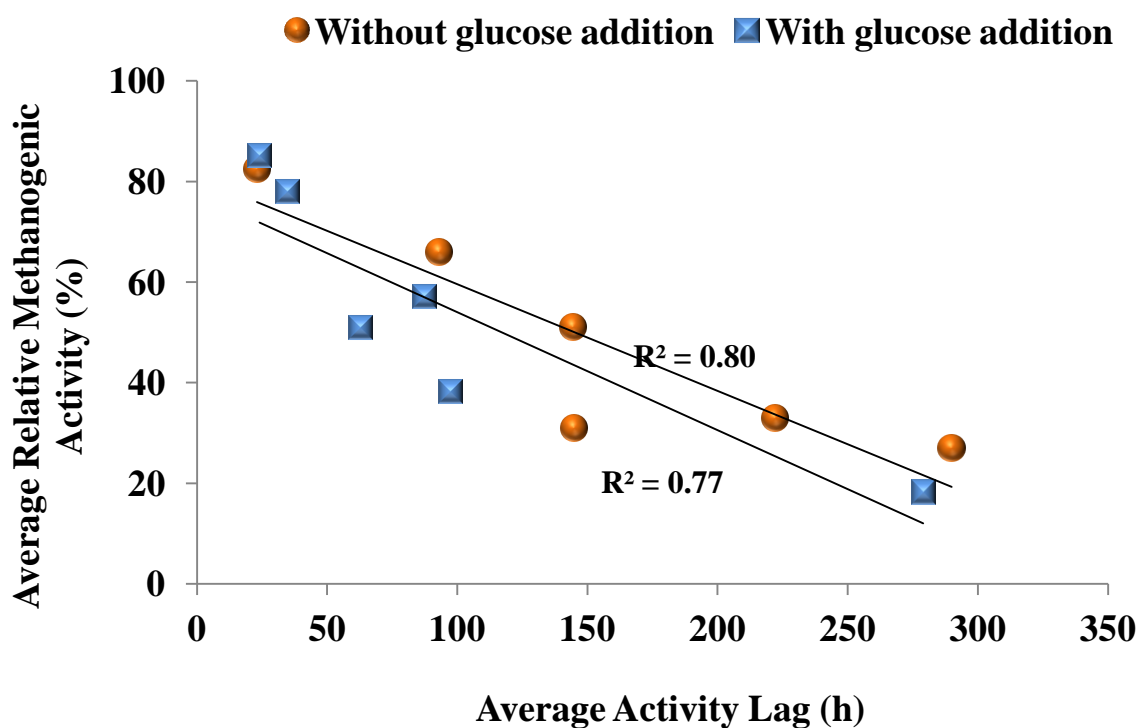
Figure 3.5. Relationship between average relative methanogenic activity and average activity lag of all preserved hydrogen-utilizing methanogenic cultures

3.3.5   Methanogenic activity over five months of storage

The relative methanogenic activity preserved over five months of storage was determined for heat-dried cultures 3 and 4 stored at 4 ^{0}C. The relative methanogenic activity retained by heat dried cultures 3 and 4 was 46 ±5.4% and 59 ±5.8% over five months (Figure 3.6). Culture 4 retained 9% greater relative methanogenic activity than culture 3. The rate of relative methanogenic activity loss for culture 3 and 4 was statistically higher for the first two months of storage and gradually decreased over subsequent period. The rate of

relative methanogenic activity loss for cultures 3 and 4 was 4.4% and 4.5% per month

over the first two months, whereas it was 2.7% and 2.8% per month averaged over the

entire five months of storage. The average activity lag after five months of storage was

337 ± 86 and 345 ± 109 h for heat-dried cultures 3 and 4, respectively. The activity lag

for cultures 3 and 4 was linearly correlated with methanogenic activity with r^2 values of

87 and 95 %, respectively.
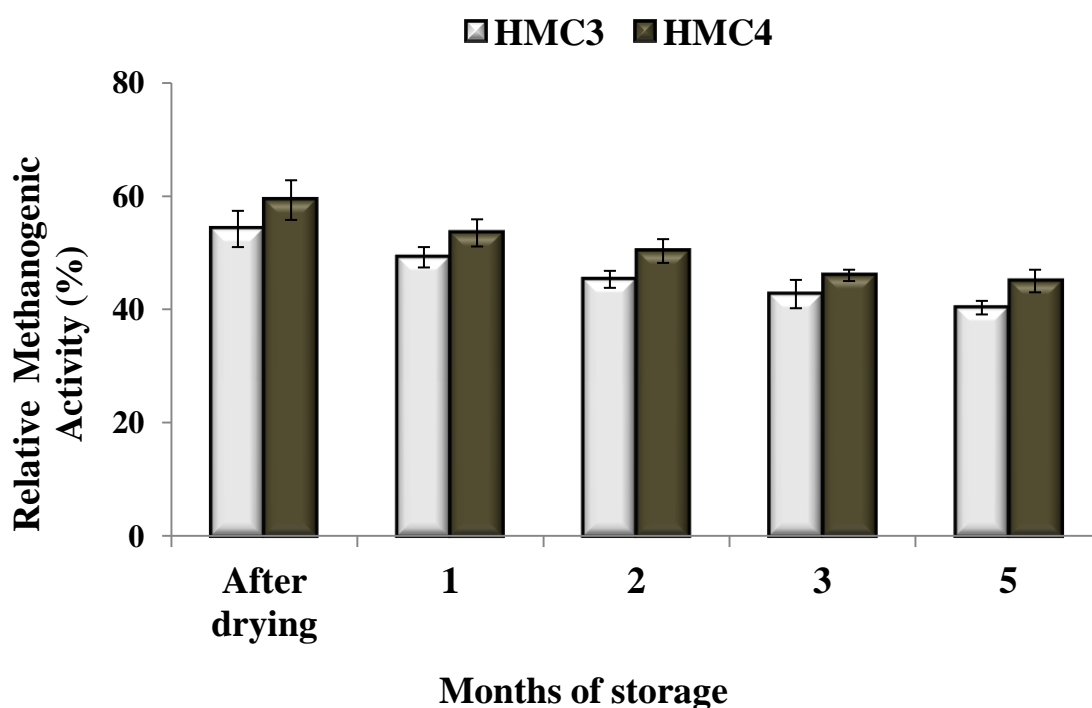
**Figure 3.6. Relative methanogenic activity preserved for heat dried
hydrogen-utilizing methanogenic cultures over months of storage**
Error bars represents the standard deviation of three replicates

3.3.6   Influence of culture growth conditions on methanogenic activity preservation

Cultures that developed under different growth conditions exhibited different responses

to different preservation methods. Culture 2 retained statistically higher relative

methanogenic activity after heat drying than culture 1. The results were opposite in the

case of freeze drying for which culture 1 exhibited higher methanogenic activity than culture 2. Furthermore, cultures 3 and 4 retained statistically higher relative methanogenic activity than cultures 1 and 2 respectively, after preservation as well as after preservation and simulated long-term storage regardless of the different preservation methods employed.

3.4   Discussion

All hydrogen-utilizing methanogenic cultures developed under different growth conditions were able to retain methanogenic activity using all preservation methods, even after handling, preserving and storing the biomass in the presence of air. Revival of significant methanogenic activity after heat and freeze drying in air confirmed that some methanogens can tolerate drying under extreme temperature under oxic condition.

All preserved cultures maintained significant methanogenic activity after simulated long-term storage as well as five months of actual storage in the presence of air. This unusual tolerance of methanogens to O_2 was not expected, especially when long-term exposure is typically considered to be lethal to methanogens (Yuan et al., 2009; Liu et al., 2008; Fetzar et al., 1993). On the contrast, methanogens in dried upland soils, desert soils and paddy soils exposed to air revealed CH_4 production after seasonal anoxic conditions returned (Fetzer et al., 1993; Ueki et al., 1997; Angel et al., 2011; Angel et al., 2012). Ueki et al. (1997) estimated 25 % of methanogens remained viable in air-dried paddy soil even after two years of storage in air. However, the preservation of acitivty of methanogenic culture using different drying techniques in the presence of air is so far not well studied. This study showed the significant activity of HMC cultures in the presence of air which could be explained with the following: 1) physiological defense

- some methanogens ability to produce O_2 detoxifying enzymes such as superoxide dismutase and catalase during oxidative stress conditions which helps them to preserve their functionality during long air exposure (Brioukhanov and Netrusov, 2007; Brioukhanov et al., 2006; Shima et al., 2001); 2) morphological defense – some methanogens possess strong cell envelopes and/or membranes than others which provides better resistance from oxic damage (Beveridge et al., 1993; Beveridge et al., 2002; Ma et al., 2006); and 3) shielding effect - some methanogens can be shielded by dead cells or non-methanogenic cells that may reduce the direct oxidative damage due to a physical barrier for O_2 diffusion (Ueki et al., 1997; Liu et al., 2008; Botheju and Bakke, 2011).

Methanogenic activity loss was found to be higher for heat drying than freeze drying and biomass centrifuging. Similar results were observed in earlier studies in which various lactic acid bacteria preserved by high temperature spray drying showed higher cell viability loss than that observed after freeze drying (Teixeira et al., 1995; To and Etzel, 1997; Wang et al., 2004). Higher process temperature during heat drying is described as a major cause of severe cell damage (Santivarangkna et al., 2008a). Furthermore, in heat drying, thermal injury occurred simultaneously with dehydration damage, whereas in freeze drying, cryo-injury occurred due to freezing prior to dehydration stress.

The rate of average methanogenic activity loss of all cultures was lower in heat-dried cultures during simulated long-term storage compared to freeze-dried and centrifuged cultures. Higher stability of heat-dried cultures compared to freeze-dried and centrifuged cultures was related to lower RMC after preservation.
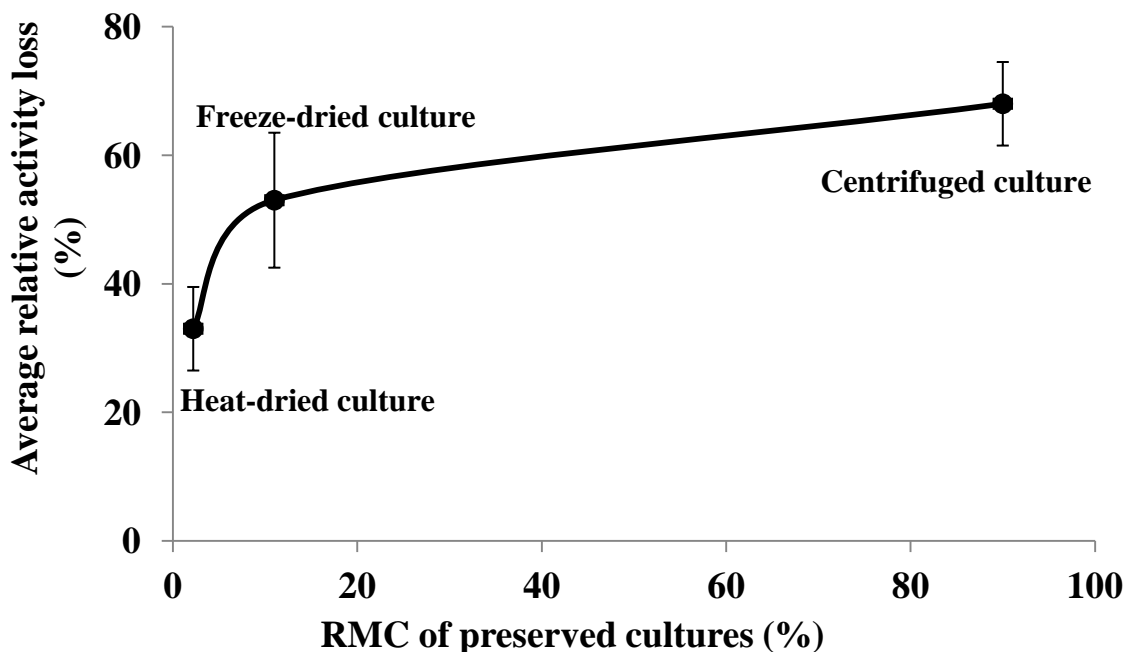
Figure 3.7. Average relative methanogenic activity loss of preserved hydrogen-utilizing methanogenic cultures during 15 days of simulated long-term storage
Error bars represent the standard deviation of twelve replicates

Previous literature demonstrated that higher RMC or water activity in preserved biomass led to greater cell viability loss during storage (Aguilera and Karel, 1997). Although low RMC results in higher stability during long-term storage, it may also lead to higher cell membrane ridigity which can cause cell leakage during rehydration, resulting in significant cell loss (Santivarangkna et al., 2008a). The optimum RMC is different for different species and needs to be determined individually. Researchers reported that RMC in the range of 2.8 to 5.6% (mass basis) was found to be optimum to preserve the activity of freeze- and spray-dried lactic acid bacteria (Zayed and Ross, 2004; Gardiner et al., 2002; Desmond et al., 2002). Typically, an RMC value less than 10% is considered to result in stability during storage (Aguilera and Karel, 1997)**.** Heat-dried culture with 2.5% RMC was found to be more stable during simulated long-term

storage than both the freeze dried culture at 11% RMC or centrifuged culture at a RMC of 91% (Figure 3.7). Significant activity loss in stored centrifuged culture was ostensibly due the higher RMC. Higher metabolic activity and endogenous decay with high RMC may have resulted in higher cell viability loss. In addition, growth of a milky, white layer formed on the centrifuged biomass surface during storage which could be fungus, may have contributed to further methanogenic activity loss. Castro et al. (2002) reported higher instability of anaerobic biomass during two months of refrigerated storage compared to frozen and freeze-dried biomass at 4 ^{0}C.

In addition to RMC, storage temperature and oxidative stress could have been considerable causes of significant activity loss after simulated long-term storage. Increased oxidation of cell membrane lipids, as measured by changes in the cell membrane fatty acid profiles, has been described to decrease cell membrane fluidity and increase membrane leakage during rehydration (Teixeira et al., 1996; Castro et al., 1996; Borst et al., 2000; Santivarangkna et al., 2008a). Furthermore, free radicals formed during oxidation of biological molecules have been suggested to cause loss of cell viability or functionality (van de Guchte et al., 2002; Santivarangkna et al., 2008a)**.** However, methanogenic activity preserved after simulated long-term storage was considered equivalent to six years of storage at 4 ^{0}C which was found to be stable for another 14 years without any further loss (Sakane and Kuroshima, 1997).

Significant methanogenic activity was preserved over five months of actual storage at 4 ^{0}C in air. A decrease in the methanogenic activity was observed during the first two months of storage; thereafter methanogenic activity loss was very low. Similar observations were made by other researchers: higher cell loss occured during the early

storage period. This may have beeen due to rapid loss of injured cell immediately after drying (Miyamoto-Shinohara et al., 2000; Corcoran et al., 2004).

The effectiveness of glucose as a protectant varied with different preservation methods. The protective effect of glucose was evident for freeze drying, but not for the other preservation methods. Strasser et al., (2009) found similar results for glucose and other protective agents when lactic acid bacterial strain *Enterococcus faecium* was freeze-dried and fluidized-bed dried at a 37 ^{0}C outlet temperature. The protective effect of glucose was greater for freeze drying than for fluidized bed drying. The lack of an observed protective effect of glucose after heat drying could be due to different process conditions and most likely due to higher process temperature. There are several factors that can affect the efficacy of protective agents including temperature (Hubalek, 2003). Previous studies suggested that sugars with higher glass transition temperatures (T_g) may provide a better protective effect during high-temperature drying and/or storage (Santivarangkana et al., 2008; Morgan et al., 2006). T_g is the maximum temperature at which a substance is in its solid glassy state and a subsequent increase in temperature results in the start of crystallization. Sugar solutions with higher viscosity possess higher T_g values. Desmond et al. (2002) used gum acacia, a highly viscous oligosaccharide, to preserve higher viability of a *Lactobacillus* strain that was exposed to high air temperatures (inlet,170 ^{0}C and outlet,105 ^{0}C) during spray drying. Santivarangkna et al. (2008) summarized that the protective capacity to stabilize cultures follows the decreasing order of T_g (trehalose 107 ^{0}C > maltose 91 ^{0}C > sucrose 67 ^{0}C > glucose 36 ^{0}C).

The protective effect of glucose was more pronounced after preservation and simulated long-term storage than preservation alone. Similar results were reported by Colleran et al. (1992) who found that 10% glucose addition to granular anaerobic biomass resulted in 32% higher hydrogenotrophic methanogenic activity after freeze drying and 49% higher activity after seven months of storage at 4 ^{0}C under anaerobic conditions. The ability of sugars to form glass matrices to minimize molecular activities, their viscosity restriction for O_2 diffusion, and protection from sudden shock of pressure imbalance during rehydration could be the major reasons for higher preservation of dried cell after simulated long-term storage in the presence of glucose (Santivarangkana et al., 2008). Higl et al. (2007) demonstrated that cell inactivation rates were lower in the glassy state and inactivation was largely affected by the storage temperature and water activity under the non-glassy state. In contrast, Strasser et al. (2009) found glucose to be inferior to sucrose and trehalose for preserving the viability of freeze- and fluidized-bed-dried lactic acid bacteria stored at 35 ^{0}C for one month. The reason for poor performance of glucose was related to its lower T_g value (36 ^{0}C) which is just above the elevated storage temperature of 35 ^{0}C and it might not have been stable during storage. However, results presented herein showed that glucose addition was effective during simulated long-term storage at 35 ^{0}C. Ohtake et al. (2004) discussed that sometimes the T_g value of sugars is altered by other salts presents in the matrix. The T_g value of trehalose and sucrose in the presence of potassium phosphate increased by 25 and 50 ^{0}C, respectively. Residual salts present in the HMC biomass suspension may have increased the T_g of glucose which resulted in better stabilization of dried biomass during simulated long-term storage at 35°C (temperature above the glucose T_g value).

A significant lag was observed in methanogenic activity of preserved cultures compared to a negligible lag in cultures before preservation. The activity lag was ostensibly a recovery and growth time to attain a significant methanogenic population that had decreased during preservation and storage. Activity lag is an important parameter to measure along with activity, and represents a measure of damage to the culture. This is valuable information needed when preserved cultures are applied to some continuously operating reactors where the residence time of preserved cultures is limited by the reactor detention time.

Preservation of HMC biomass developed under different growth conditions varied among the different preservation methods employed. For example, culture 2 developed with glucose in the feed retained higher relative methanogenic activity after heat drying compared to culture 1 that was not fed glucose; results were opposite for freeze drying. Furthermore, cultures 3 and 4 developed in micro-oxic conditions retained higher methanogenic activity after preservation, and after simulated long-term storage compared to their respective cultures 1 and 2 developed under anaerobic conditions. The methanogenic communities developed under glucose growth conditions might be more tolerant to heat drying and more sensitive to freezing drying. Micro-oxic conditions during culture growth may result in methanogenic communities that have the higher tolerance to O_2 exposure during preservation and subsequent storage.

3.5   Conclusion

Preservation and long-term storage of methanogenic cultures is possible using high or low temperature drying in presence of air during handling, drying and storage. Hydrogen-utilizing methanogenic cultures are able to tolerate high desiccation and heat stress and

subsequent storage in am air atmosphere. The dried cultures exhibited significant activity after rehydration. Even though methanogens are considered to be sensitive organisms, at least some species are more resilient to the high stress of drying, heating and air exposure than previously assumed.

Heat drying imposed higher methanogenic activity loss compared to freeze drying and centrifuging. However, heat-dried cultures were more stable during simulated long-term storage than cultures preserved by other methods. This corresponded to lower RMC values for heat-dried cultures than for freeze-dried and centrifuged cultures. Centrifuged cultures were found to be highly unstable during simulated long-term storage.

The effectiveness of glucose addition as a protective agent varied among the different preservation methods and the effectiveness was more profound for freeze drying than for heat drying. Criteria for protectant selection on a commercial scale need to be based on protectant availability, cost, and the excess COD exerted due to the additive when introduced in an anaerobic system.

Preservation and subsequent storage of methanogenic culture is influenced by culture growth conditions. Cultures developed with a small amount of glucose showed higher preservation after heat drying, whereas culture developed with no glucose showed better preservation after freeze-drying. Furthermore, cultures exposed to O_2 during enrichment exhibited higher methanogenic activity after preservation and subsequent storage regardless of the preservation method employed.

This work was an initial study of commercial methanogenic culture preservation and long-term storage in an air atmosphere. Future research is suggested to improve the methanogenic activity and/or to reduce activity lag of preserved cultures by optimizing

the process parameters of enrichment and preservation methods, different storage

conditions, and different protective agents.

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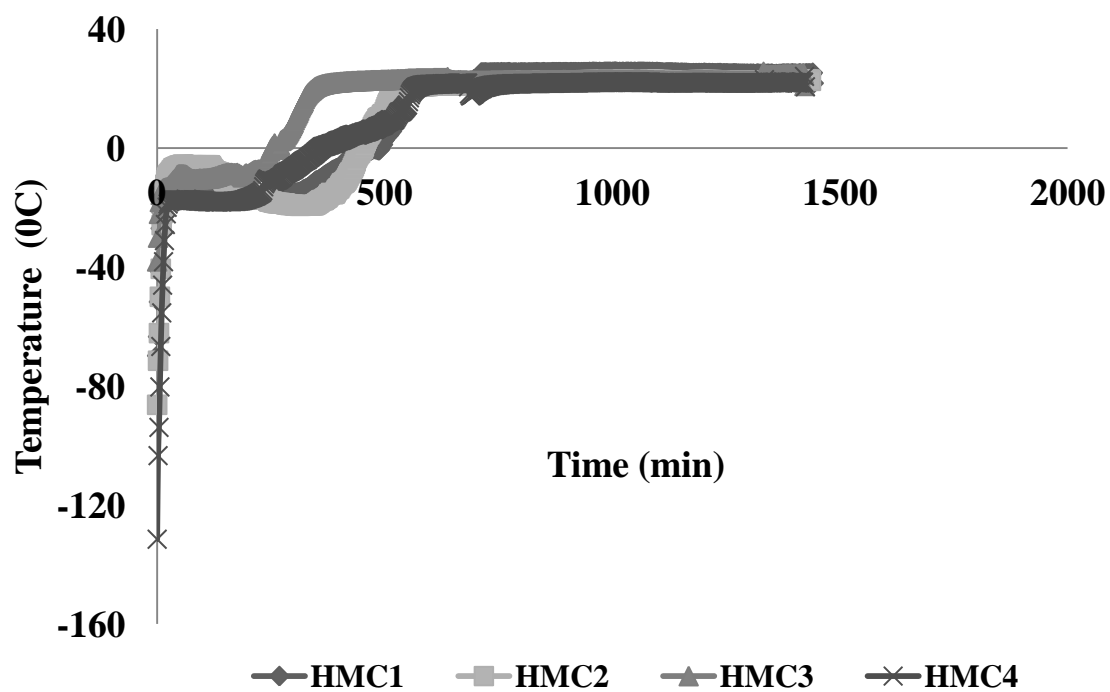
3.7   Appendix II

Figure 3.8. Freeze drying behaviour of hydrogen-utilizing methanogenic cultures

Chapter 4 Micro-Oxic Growth Condition Enhanced Preservation of H$_2$-utilizing Methanogenic Culture in Presence of Air

4.1   Introduction

In anaerobic biotechnology, methanogenesis is the terminal step converting complex organic waste into CH$_4$. It plays a crucial role in efficient performance of anaerobic bioprocess by maintaining a low H$_2$ concentration to drive thermodynamically unfavorable fermentation reactions (McCarty and Smith, 1986). Therefore, preservation of methanogenic cultures for various research and practical applications would be beneficial. Preserved methanogenic biomass can be used as starter culture to seed or re-seed laboratory as well as full scale reactors. It can be employed for bioaugmentation purposes to enhance anaerobic digestion. It can also be used as a defined culture for various standard laboratory tests.

Preservation of cultures by drying has been a preferred method for cost effective shipping and handling, and long-term storage (Santivarangkna et al., 2007; Peighambardoust et al., 2011). However, preservation of significant microbial activity by drying can be challenging. Drying imposes severe stress on microbial cells which can cause significant damage to cellular components and protein and can lead to permanent viability loss (Potts, 2001; Franca et al., 2007; Santivarangkna et al., 2008).

One suggested method to increase cell survivability during drying and storage is to induce a stress during culture growth (Carvalho et al., 2004; Morgan et al., 2006; Santivarangkna et al., 2007). Previous studies have shown increases in the cell viability after drying and subsequent storage by exposing cultures to stressful conditions such as low pH, (Palmfeldt and Hahn-Hagerdal, 2000), heat and cold shock (Maus and Inghm, 2003; Santivarangkna et al., 2007) and short-term starvation (Carvalho et al., 2003;

Morgan et al., 2006). Higher cell survivability after drying and storage is attributed to higher tolerance responses induced in cells due to their stressful growth conditions.

Methanogens are categorized as strict anaerobes and considered to be highly sensitive to oxygen toxicity. The presence of oxygen can inhibit methanogen growth by damaging critical cell components such as the cell membrane, proteins and DNA (Schonheit et al., 1981; Jarrell, 1985; Brioukhanov and Netrusov, 2007). Members of the *Methanococcus* genera showed almost zero tolerance to O_2 (Kiener and Leisinger, 1983), whereas some methanogens from the *methanobacterium, methanosarcina* and *methanobrevibcator* genera demonstrated limited tolerance to O_2 (Liu et al., 2008; Botheju and Bakke, 2011). Some methanogens synthesize O_2-detoxifying enzymes as a defensive mechanism to tolerate limited oxidative stress (Brioukhanov, et al., 2002; Brioukhanov et al., 2006; Brioukhanov et al., 2007; Kratzer et al., 2011).

Literature on preservation of methanogenic cultures using drying is limited and preservation is typially performed at laboratory scale under strict anaerobic conditions (Castro et al., 2002; Cleland et al., 2004; Iino and Suzuki, 2006). However, preservation of methanogenic cultures under strict anaerobic conditions is an expensive option for commercial-scale production. It is almost impractical to maintain strict anaerobic conditions during handling, preservation and storage of methanogenic biomass. Preservation of microbial cultures using freeze drying is a well accepted method for preserving various microorganisms, including anaerobes (Staab and Ely, 1987; Malik, 1990; Cleland et al., 2004; Morgan et al., 2006).

In this study, we hypothesized that methanogenic cultures growth under micro-oxic conditions can preserve higher methanogenic activity after freeze drying and after its subsequent during long-term storage in presernce of air.

4.2   Methods

4.2.1   Culture development

Development of the H_2-utilizing methanogenic culture (HMC) was described in detailed by Schauer-Gimenez et al. (2010). In brief, the HMC cultures were developed on two different growth media. HMC1 was developed on H_2:CO_2 (1:1, v/v ratio) as a substrate, whereas HMC2 was developed on H_2:CO_2 (1:1, v/v ratio) and glucose (40 mg/L-day) as a substrate. Both cultures were developed under two conditions: strict anaerobic and micro-oxic. The micro-oxic condition was maintained by adding O_2 (40 mg O_2/L-day) in the form of air to reactor headspace which satisfied less than 6% of the theoretical oxygen demand. All cultures were developed in 2 L laboratory bioreactors seeded with mesophillic municipal biomass and operated at 35 ^{0}C and a 15-day retention time over 2 years. Substrate and basal nutrient addition was carried out in feed-and-draw mode once a day as discussed below by others (Speece, 2008; Schauer-Gimenez et al., 2010).

Basal nutrient medium contained the following [mg/L]: NH_4Cl [400]; $MgSO_4 \cdot 6H_2O$ [250]; KCl [400]; $CaCl_2 \cdot 2H_2O$ [120]; $(NH_4)_2HPO_4$ [80]; $FeCl_3 \cdot 6H_2O$ [55]; $CoCl_2 \cdot 6H_2O$ [10]; KI [10]; yeast extract [100]; the trace metal salts $MnCl_2 \cdot 4H_2O$, NH_4VO_3, $CuCl_2 \cdot 2H_2O$, $Zn(C_2H_3O_2)_2 \cdot 2H_2O$, $AlCl_3 \cdot 6H_2O$, $NaMoO_4 \cdot 2H_2O$, H_3BO_3, $NiCl_2 \cdot 6H_2O$, $NaWO_4 \cdot 2H_2O$, and Na_2SeO_3 [each at 0.5]; $NaHCO_3$ [5000]; and resazurin [1].

4.2.2 Culture preservation and simulated long-term storage

Culture preservation and its simulated long-term storage were performed as described in Chapter 3. In brief, the culture was freeze-dried by freezing in liquid nitrogen for 7 min in presernce of air and subsequently dried under vacuum using a bench-top freeze dryer (3GenMP Opti-Dry, Millrock Technology, Kingston, NY, USA) at -45 ^{0}C condenser temperature and 13.3 Pa pressure for 24 h (Staab and Ely, 1987; Porbucan, 1991; Simione and Brown, 1991). The preserved culture was temporarily stored for 2 days at room temperature (20-23 ^{0}C) prior to activity testing after drying. For long-term storage, the preserved culture was stored for 15 days at 35 ^{0}C which has been shown to simulate 20 years of storage at 5 ^{0}C under vacuum for many microorganisms (Sakane and Kuroshima, 1997). The preserved culture was placed in a desiccator containing a $CaSO_4$ dessicant (Drierite$^®$, W. A. Hammond Co., Xenia, OH, USA) during storage. Culture handling, drying and storage were carried out in the presence of air.

4.2.3 Methanogenic activity of preserved cultures

SMA is a measure of maximum specific CH_4 production rate per gram of biomass and expressed as mL CH_4/gVSS-h. The SMA values of dried and non-dried cultures were determined and compared using H_2 and acetate as substrates. SMA testing for H_2:CO_2 and acetate was performed according to the protocols developed by Coates et al. (1996) and Anagelidaki et al. (2007), respectively, and described in detailed in previous Chapter 3.

4.2.4 Quantification of methanogenic community

The methanogenic community was quantified using reverse transcription quantitative-PCR (RT-qPCR) for *mcrA*-specific primers. The procedure for nucleic acid isolation,

reverse transcription-PCR (RT-PCR) and quantitative PCR (qPCR) reactions was performed as described in Chapter 2.

Nucleic acid extraction: RNA was isolated from non-dried and dried culture (after reconstitution) in duplicate using an RNA PowerSoil™ Total RNA Isolation Kit and DNA Elution Accessory Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) as per the manufacturer's protocol. Isolated RNA was further treated with RNase-free DNase and purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as per the recommended protocol. The integrity and purity of isolated products were analyzed on agarose gels (1.5% w/v) stained with ethidium bromide and quantified using a spectrophotometer (Nanodrop ND-1000, ThermoScientific, Waltham, MA, USA).

RT-PCR: From the isolated RNA pool, 1200 ng of RNA was synthesized for its complimentary DNA (cDNA), using a *mcrA* reverse primer (Luton et al., 2002) and other reagents provided with the iScript® Select cDNA Synthesis Kit (Biorad, Hercules, CA, USA) and following the manufacturer's protocol. The quality of the analysis was controlled by including a no-template and a no-reverse-transcriptase control for each sample.

qPCR: All qPCR reactions were performed using a Biorad My iQ single color RT-PCR detection system. The qPCR was performed with *mcrA* gene-specific primers described elsewhere (Luton et al., 2002).The sample of 40ng/µL cDNA was standardized for all qPCR reactions and run in duplicate for each sample. The final qPCR mix per 25 µl reaction was as follows: 1X iQ™ SYBR® Green Supermix reaction buffer containing dNTPS , iTaq DNA polymerase and 3 mM $MgCl_2$ (Biorad, Hercules, CA, USA); and template cDNA and *mcrA* primers 750 nmol/L. The qPCR reactions proceeded as

follows: initial denaturation at 95^0C (10 min), followed by 45 cycles of $95\ ^0$C (30 s), 58.5

(60 s) and a final extension at $72\ ^0$C (7 min). The amplification was followed by a

denaturation curve program (80 cycles 10 sec in length starting at $55\ ^0$C and increasing in

$0.5\ ^0$C increments) to check for product specificity. *mcrA* gene and transcript copies were

quantified by constructing a standard curve with each qPCR run. The standard curve was

developed as described elsewhere (Morris, 2011). Starting quantity amounts and

threshold cycle values were calculated using the MyiQ™ optical system software version

1.0.

4.2.5   Analysis

Data were analyzed using the SPSS statistical program (Statistic Base 17.0). An analysis

of variance was performed on the data and Tuckey's honestly significant difference

(HSD) statistic was used for multiple comparisons of means at a 5% significance level.

The one-sided Student's t-test with unequal population variance at a 95% confidence

level was performed for statistical comparison between two sets of samples.

The transcript copies were estimated using the formula discussed elsewhere (Yu

et al., 2005) and modified with regards to biomass concentration.

1) Transcript copies/gVSS

$$= \frac{\text{RNA concentration } \left(\frac{\text{gRNA}}{\text{mL}}\right) \times 6 \times 10^{23} \left(\frac{\text{copy}}{\text{mole}}\right)}{\text{Biomass concentration } \left(\frac{\text{gVSS}}{\text{mL}}\right) \times mcrA \text{ amplicon size (bp)} \times 330 \left(\frac{\text{g}\frac{\text{RNA}}{\text{mole}}}{\text{bp}}\right)} \dots \text{Eqn 1.}$$

2) Gene copies/gVSS

$$= \frac{\text{DNA concentration} \left(\frac{\text{gDNA}}{\text{mL}}\right) \times 6 \times 10^{23} \left(\frac{\text{copy}}{\text{mole}}\right)}{\text{Biomass concentration} \left(\frac{\text{gVSS}}{\text{mL}}\right) \times mcrA \text{ amplicon size (bp)} \times 660 \left(\frac{\text{g} \frac{\text{RNA}}{\text{mole}}}{\text{bp}}\right)} \ldots \text{Eqn 2.}$$

3) Percent methanogenic activity (%)

$$= \frac{\text{Average SMA of HMC biomass after drying or storage}}{\text{Average SMA of HMC biomass before drying}} \times 100 \ldots \ldots \text{Eqn 3.}$$

4) Percent $mcrA$ transcript or gene copy (%)

$$= \frac{\text{Average } mcrA \text{ transcript or gene copies/gVSS after drying or storage}}{\text{Average } mcrA \text{ transcript or gene copies/gVSS before drying}} 100 \ldots \ldots \text{Eqn 4.}$$

4.3   Results and Discussion

Cultures HMC1 and HMC2 developed under anaerobic and micro-oxic conditions, respectively, demonstrated significant methanogenic activity before and after freeze drying as well as after simulated long-term storage (Figure 4.1a). The cultures exhibited methanogenic activity for H_2, whereas negligible activity was seen for acetate.

The methanogenic activities of cultures developed on different growth media were statistically different. HMC1 developed on H_2:CO_2 maintained significantly higher methanogenic activity than HMC2 developed on H_2:CO_2 and glucose. The HMC1 culture developed under micro-oxic conditions maintained statistically higher methanogenic activity ($p > 0.05$) before and after freeze drying, and after simulated long-term storage compared to the culture developed under strict anaerobic conditions. In contrast, the HMC2 culture developed under micro-oxic conditions had statistically lower

methanogenic activity than culture developed under strict anaerobic conditions before and after freeze drying, and no statistical difference was found in the activities of micro-oxic and strict anaerobic cultures after simulated long-term storage. However, the percent methanogenic activity of cultures developed under micro-oxic conditions was significantly higher after freeze drying, and after subsequent simulated long-term storage than the culture developed under anaerobic conditions regardless of the culture growth medium (Figure 4.2a). The HMC1 developed in micro-oxic conditions retained 3.5% higher activity after freeze drying ($p < 0.05$), and 83% higher activity after simulated long-term storage ($p > 0.05$) compared to the strictly anaerobic culture. Similarly, the HMC2 developed in micro-oxic conditions retained 8% higher methanogenic activity after freeze drying ($p > 0.05$) and 64% higher after simulated long-term storage ($p > 0.05$) compared to the strictly anaerobic culture. Higher methanogenic activity retention of cultures developed under micro-oxic conditions could be due to the development of a tolerant methanogenic community that is responsive to handle stressful conditions, such as oxic stress, during drying and subsequent long-term storage. The production of detoxifying proteins such as superoxide dismutase and catalase under micro-oxic conditions might have reduced cell damage during drying and simulated long-term storage.

Quantification of *mcrA* transcripts and gene copies per gram of VSS supported the methanogenic activity results (Figures 4.1 b and 4.1c). Methanogenic activity was linearly correlated 93% with the natural log of transcript copy number ($r^2 = 0.93$), and with the natural log of gene copy number ($r^2 = 0.70$) (Figure 4.3) and the slope represented the rate of methne production per hour per transcript or gene copies. The HMC cultures developed under micro-oxic conditions showed a higher number of transcript and gene

copy values after drying and after simulated long-term storage compared to the culture developed under anaerobic condition (Figures 4.2 b and 4.2c). The HMC1 culture developed under micro-oxic conditions contained 52% (p> 0.05) and 68% (p> 0.05) higher transcript copies after freeze drying, and after simulated long-term storage, respectively, compared to the anaerobic culture. Likewise, the HMC 2 culture developed under micro-oxic conditions demonstrated 43% (p> 0.05) and 155% (p> 0.05) greater numbers of transcript copies after freeze drying, and after simulated long-term storage, respectively, compared to the anaerobic culture. A similar pattern was observed for *mcrA* gene copy numbers.
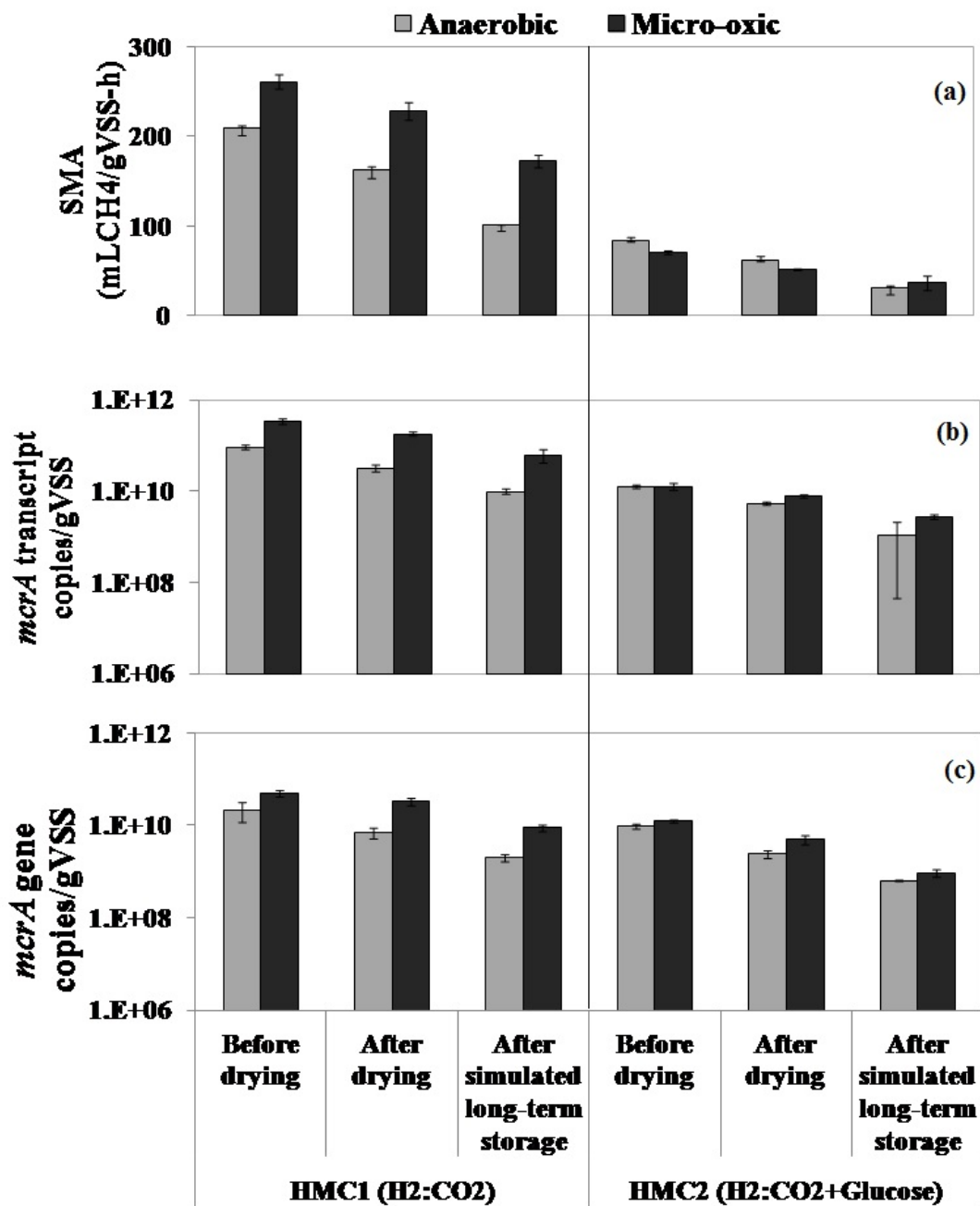
Figure 4.1. Methanogenic activity and *mcrA* transcript and gene copies of hydrogen-utilizing methanogenic cultures before and after freeze drying, and after simulated long-term storage
Error bars represent the standard deviation of three replicates for (a), and standard deviation of four replicates for (b) and (c). Some error bars are small and not visible.
The work was done collaborativly with Keerthi Cherukuri (Department of Biological Science, Marquette University)
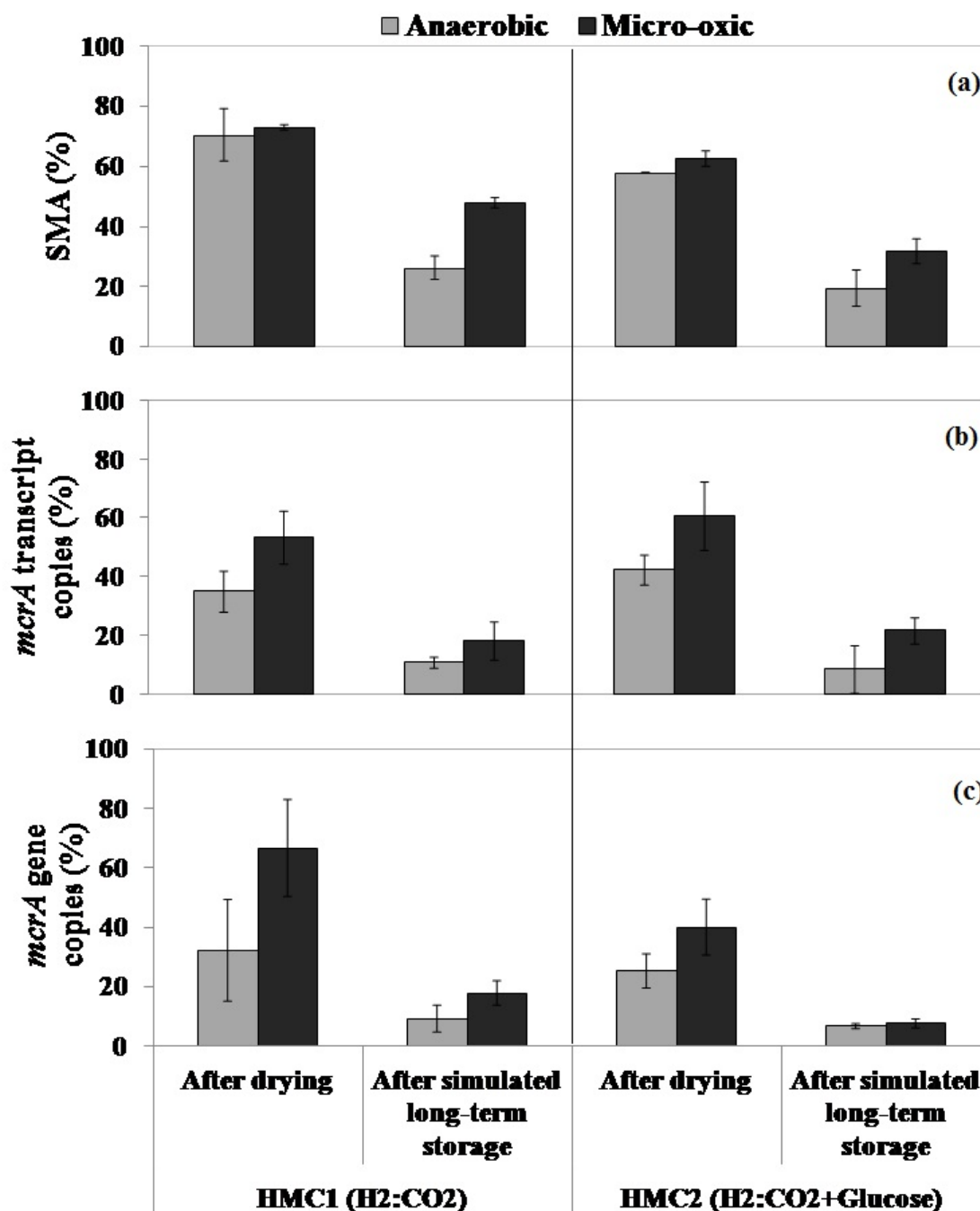
Figure 4.2. Percent Methanogenic activity, and *mcrA* transcript and gene copies of hydrogen-utilizing methanogenic cultures before and after freeze drying, and after simulated long-term storage
Error bars represent the standard deviation of three replicates for (a), and standard deviation of four replicates (b and c). Some error bars are too small and not visible. The work was done collaborativly with Keerthi Cherukuri (Department of Biological Science, Marquette University)

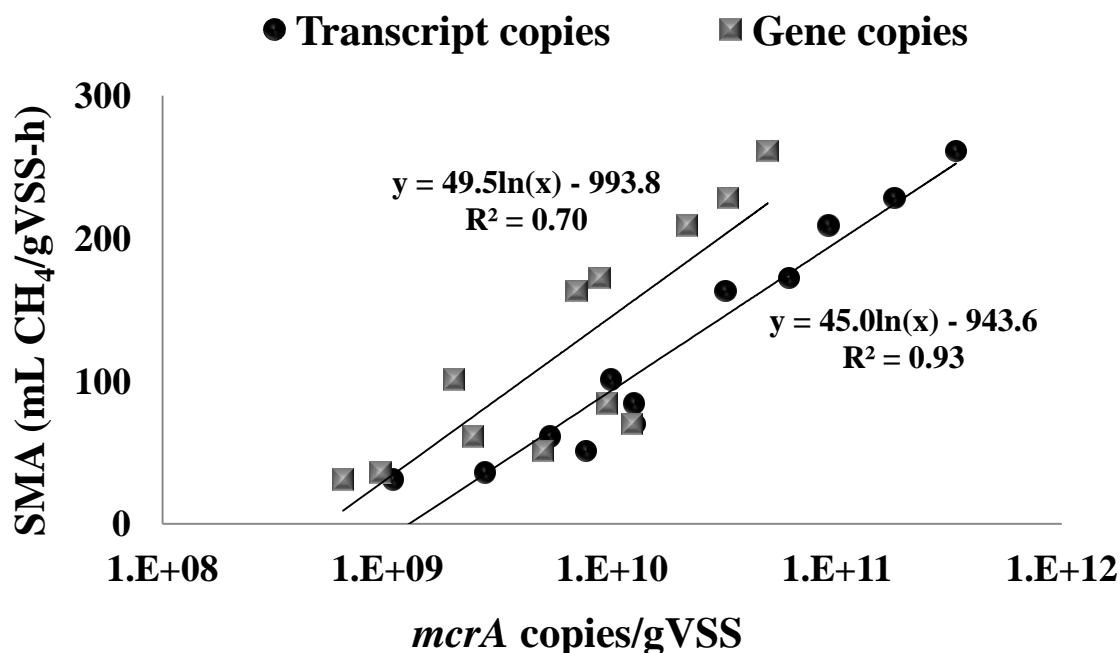**Figure 4. 3. Relationship between average specific methanogenic activity and mcrA transcript and gene copies of hydrogen-utilizing methanogenic cultures before and after freeze drying and after simulated long-term storage of the cultures**

4.4   Conclusion

A culture under micro-oxic conditions retained higher methanogenic activity after drying,

and after simulated long-term storage in air compared to a culture developed under strict

anaerobic conditions. The qPCR results on *mcrA* transcript copies supported the

methanogenic activity results.

4.5   References

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Chapter 5 Preserved, Dried Methanogenic Biomass for Bioaugmentation and Standard Laboratory Tests: A Novel Approach

5.1 Introduction

Anaerobic digestion is a well established technology for treatment of high-strength organic wastewater (Rajeshwari et al., 2000; Chan et al., 2009; Ersahin et al., 2011). However, process stability is a key for successful operation of this technology (Leitao et al., 2006). A modest increase in digestion performance can save significant operating costs by decreasing aerobic post treatment costs and increasing CH_4 production for renewable energy.

One possible method to improve process performance and stability of biological systems is bioaugmentation (Nyer and Bourgeois, 1980; Parker and Wanner, 2007). Bioaugmentation involves the addition of specialized or adapted microorganisms to increase the overall metabolic capabilities of native micro flora present in the biological system. Bioaugmentation has traditionally been used to remediate sites contaminated with toxic chemicals (Vogel, 1996; Deflaun and Steffan, 2002; Gentry et al., 2004). More recently, it has been applied to aerobic and anaerobic wastewater treatment to improve process performance and stability when wastewater COD removal proceeds slowly due to poor metabolic capabilities and/or slow adaptation of native microorganisms (Mohan et al., 2005; Mohan et al., 2007; Xu et al., 2008; Lenz et al., 2009). Bioaugmentation using nitrifying bacteria has been found to enhance the nitrification process of poorly performing, unstable aerobic wastewater treatment systems (Rittmann and Whiteman, 1994; Abeysinghe et al., 2002; Salem et al., 2003; Head and Oleszkiewicz, 2004; Parker and Wanner, 2007; Xu et al., 2008; Leu and Stenstrom, 2010).

In anaerobic system, methanogens converts organic COD in the form of the intermediates H_2 and acetate into CH_4 in the terminal step of methanogenesis. This step is often the rate-limiting step to convert soluble organic compounds to CH_4 due to methanogen's slow kinetics compared to fermentative bacteria (Lyberatos and Skiadas, 1999; Ribes et al., 2004). The rapid utilization of fermentative products, especially H_2 and acetate, makes the process efficient and stable. Therefore, bioaugmentation using methanogenic biomass that degrades H_2 and acetate could be a beneficial approach to expedite the biochemical process.

Previous studies have shown the benefits of bioaugmentation using methanogenically active biomass to increase the degradation rate of difficult-to-degrade halogenated and aromatic compound (Charest et al., 1999; Hajji et al., 1999; Hajji et al., 2000; Da Silva, 2004), increase CH_4 production in pschycrophillic bioreactors (Qiu et al., 2011), reduce the start up time of digesters (Guiot et al., 2000; Saravanane et al., 2001), and shorten digester recovery time after organic overload (Lynch et al., 1987; Tale et al., 2011). Schauer-Gimenez et al. (2010) discussed both the failure and success of bioaugmentation to reduce recovery time of transiently upset digesters due to a toxic event. They concluded that more research is needed to understand the efficacy of bioaugmentation in these systems. Progressive understanding of the relationship between anaerobic microbiology and digester functionality could help to develop customized methanogenic cultures that could be more efficient for specific anaerobic applications. Engineered, methanogenic biomass could be used for standardization of anaerobic laboratory tests, such as the biochemical methane potential (BMP) and anaerobic toxicity assay (ATA).

It would also be beneficial if active, freeze-dried methanogenic biomass (FDMB) was available to improve full-scale anaerobic treatment systems or to perform standard laboratory tests in the same way the freeze-dried aerobic microbes are commercially available to enhance nitrification or as inoculums for BOD testing (e.g., Polyseed®). Most of the information on bioaugmentation using dried cultures is limited to manufacture's literature. Cost-effective shipping and handling, easy availability and distribution, and stability of dried biomass during storage (Aguilera and Karel, 1997) would encourage development and full-scale production of various methanogenic cultures for commercial and research purposes. However, use of FDMB in anaerobic biotechnology is presently rare due to difficulties preserving the activity of methanogenic cultures and uncertainty regarding the effectiveness of bioaugmentation.

In the work reported herein, a novel approach was used to preserve a methanogenically active biomass. The resulting FDMB was used to successfully bioaugment upset lab-scale digesters as well as to perform standard anaerobic laboratory tests.

5.2   Methods
5.2.1   Culture development and freeze drying

Culture development and freeze drying were described in Chapter 2. In brief, methanogenically active biomass was developed using a laboratory anaerobic complete-mix stirred tank reactor (CSTR) fed 2.7 gCOD/L-d non-fat dry milk and essential nutrient medium (as described below) at $35 \pm 2\ {}^{0}C$ and a 10-day retention time over 3 years. Methanogenic biomass was freeze-dried by first freezing the biomass in liquid nitrogen and subsequent drying using a bench-top freeze dryer (3GenMP Opti-Dry, Millrock Technology, Kingston, NY, USA) at $-45\ {}^{0}C$ condenser temperature and 13.3 Pa pressure

for 24 h (Staab and Ely, 1987; Porubcan, 1991; Simione and Brown, 1991). The FDMB was transferred to glass chromatography vials (30 mL) with rubber septa and plastic closures. An aliquot of the FDMB was used for methanogenic activity and BMP testing, whereas the remaining FDMB was stored at 4 ^{0}C (Morgan et al., 2006; Miyamoto-Shinohana et al., 2007) for 2 months in a desiccator containing $CaSO_4$ dessicant (Drierite$^®$, W. A. Hammond Co., Xenia, OH, USA), prior to its use for bioaugmentation. Strict anaerobic conditions were not maintained during freeze drying or biomass storage.

Basal nutrient medium as suggested by Speece (2008), contained the following [mg/L]: NH_4Cl [400]; $MgSO_4•6H_2O$ [250]; KCl [400]; $CaCl_2•2H_2O$ [120]; $(NH_4)_2HPO_4$ [80]; $FeCl_3•6H_2O$ [55]; $CoCl_2•6H_2O$ [10]; KI [10]; yeast extract [100]; the trace metal salts $MnCl_2•4H_2O$, NH_4VO_3, $CuCl_2•2H_2O$, $Zn(C_2H_3O_2)_2•2H_2O$, $AlCl_3•6H_2O$, $NaMoO_4•2H_2O$, H_3BO_3, $NiCl_2•6H_2O$, $NaWO_4•2H_2O$, and Na_2SeO_3 [each at 0.5]; $NaHCO_3$ [5000]; and resazurin [1].

5.2.2   Methanogenic activity testing for freeze-dried biomass

Methanogenic activity was measured for H_2 and acetate using the specific methanogenic activity (SMA) test described in Chapter 3 based on the protocol suggested by others (Coates et al., 1996; Anagelidaki et al., 2007). Briefly, FDMB was reconstituted in basal nutrient medium containing L-cysteine hydrochloride (500 mg/L) as a reducing agent (Coates et al., 1996). Serum bottles (160 mL) were charged with 25 mL of biomass suspension. All bottles were sparged with O_2-free gas (7:3 v/v N_2:CO_2) and closed with air-tight solid, black butyl rubber septa (Geo-Microbial Technologies, Inc., Ochelata, OK, USA), crimped with an aluminum seal and incubated at 35 ^{0}C and 150 rpm in a shaker/incubator (C25KC, New Brunswick Scientific, Edison, NJ, USA). For the H_2

assay, 100 mL of $H_2:CO_2$ gas mix (4:1 v/v) was injected into test bottles having biomass VSS concentration ≤ 0.3 g VSS/L. For the acetate assay, 10 g/L of calcium acetate was added to test bottles containing biomass VSS concentration ≤ 2 g VSS/L. The SMA was calculated by measuring the maximum CH_4 production rate divided by total biomass mass. A similar protocol was followed for non-dried biomass. All tests were performed using four replicates.

5.2.3 Bioaugmentation effect of freeze-dried biomass

The effectiveness of adding the FDMB to improve performance and recovery time of transiently upset digesters was determined by intentionally exposing quasi steady-state digesters to a model toxicant, O_2, and bioaugmenting with the FDMB until the digesters achieved a new quasi steady-state condition. Anaerobic digesters were160-mL serum bottles seeded with 50 mL of the same methanogenically active biomass (non-dried) that was freeze dried. Digesters were fed non-fat dry milk (2.7 gCOD/L-d) and operated at 35°C and a 20-day retention time. Digester feeding was performed in feed-and-draw mode once a day using a 5-mL plastic syringe. After 4 SRTs, the digesters were exposed to O_2 by injecting 10 mL of O_2/d into the digester headspace for one week. The upset digesters were bioaugmented with FDMB at a dose of 100 mgVSS/L-d twice a week. The freeze-dried biomass was added directly into the daily feed and, therefore, the feed volume remained constant throughout the study. The bioaugmented digester results were compared to those of one set of control digesters that was never upset, a second set that was upset but not augmented, and a third set that was upset, but received autoclaved biomass in place of the active FDMB. The biomass was autoclaved for one hr at 20 psi and 120 ^{0}C. Each set was run in triplicate.

5.2.3.1 Digester monitoring

Daily biogas production was measured using a water-lubricated glass syringe via the plunger displacement method. CH_4 concentration in biogas was analyzed using gas chromatography (GC) (Model 7890A, Agilent Technologies, Santa Clara CA, USA). Helium was supplied as the carrier gas at a flow of 4.5 mL/min. The injector and detector temperatures were set at 150 ^{0}C and 250 ^{0}C respectively, whereas the oven temperature set at 40 ^{0}C. For soluble COD (SCOD) and volatile fatty acid (VFA) analysis, effluent samples from each digester were centrifuged at 10,000×g for 10 min and filtered using a 0.45 μm filter (Whatman International Ltd., Maidstone, England). The SCOD concentration of filtered samples was determined by standard methods (APHA et al., 2005). For VFA analysis, filtrated samples were acidified with 1% phosphoric acid and analyzed for acetic acid (HAc), propionic acid (HPr), iso-butyric acid (Iso-HBr), butyric acid (HBu), iso-valeric acid (Iso-HVa), and valeric acid (HVa) using GC. The GC conditions for measuring VFAs were maintained as described by standard method 5560 D (APHA et al., 2005). The pH was monitored using a bench-top pH meter and a general purpose electrode.

5.2.3.2 Microbial community analysis

Methanogenic community dynamics in all digesters during bioaugmentation were analyzed using denaturing gradient gel electrophoresis (DGGE) targeting the methyl coenzyme M reductase (*mcrA)* gene. The *mcrA* gene is unique to methanogens and codes for the α subunit of *methyl coenzyme-M reductase* (MCR), a key enzyme in CH_4 production (Luton et al., 2002). DGGE is a method to separate DNA fragments based on their G-C content which possess differential mobility through a DNA-denaturing gel (Head et al., 1998; Muyzer, 1999; Spiegelman et al., 2005; Talbot et al., 2008). DGGE

with *mcrA* as a target gene has been used to determine relationships between methanogenic community structure and anaerobic digester function (Appels et al., 2011; Kymalainen et al., 2011; Tale et al., 2011).

The protocol for DNA extraction, amplification and DGGE has been described elsewhere (Tale et al., 2011; Navaratnam, 2012). In brief, DNA was isolated from 750-µL biomass samples using the PowerSoil™ Total DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) and the manufacture's recommended protocol. Isolated DNA was PCR-amplified with *mcrA* gene specific primers, mcrF (5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3') and mcrR (5'-TTCATTGCRTAGTTWGGRTAGTT-3') designed by Luton et al., (2002) followed by nested PCR with mcrF GC-clamp as reported by Tale et al. (2011). The 100 µL PCR reaction contained 100 to150 ng of DNA template as well as EconoTaq® PLUS Master Mix 2X which included the Taq polymerase (Lucigen Corporation, Middleton, WI), 100 nM of each primer, and Nuclease-free H_2O as makeup volume. The PCR conditions were as follows: initial denaturation at 95°C (5 min), 35 cycles of 95°C (1min), 49°C (1 min), and 72°C (3 min), and a final extension of 10 minutes at 72°C. In the nested *mcrA*-PCR using the GC-clamp, the PCR conditions were the same with a single change in temperature of 58.5°C (1 min), instead of 49°C (1 min). The program included a slow ramp in temperature (0.1°C/s) between the annealing and extension steps of the first 5 cycles of the protocol to assist in the initial formation of product due to the degenerate nature of the primers, as recommended by Luton et al. (2002). The *mcrA*-PCR product was cleaned using the UltraClean™ PCR Clean-up™ kit (MoBio Laboratories, Carlsbad,

CA, USA), and the DNA concentration of cleaned product was measured using a

spectrophotomer (Nanodrop ND-1000, ThermoScientific, Waltham, MA, USA).

The guidelines for DGGE are described in detail elsewhere (Muyzer, 1999). The

mcrA-PCR product was separated using 8% polyacrylamide gel (37.5:1 acrylamide to

bis-acrylamide) with 30-70% denaturant gradient (urea and formamide). A total mix of

42 µL (*mcrA*-PCR-DNA: 800 ng in 35 µL, and 6X syber gold dye: 7 µL) was loaded in

each DGGE lane, and the gel was run for 15 h at 100 V. The DGGE gel was stained with

1% SYBR® green (Invitrogen, CA, USA) for 30 minutes and visualized using a

transilluminator (M-20, UVP, Upland, CA, USA). The optical density of each distinct

band captured in the DGGE gel image was determined using Lab Works software

(v.4.6.00.0). A common ladder was loaded with each DGGE gel to normalize the sample

optical density data. The analysis was performed on band densitometric data as described

elsewhere (Tale et al., 2011; Navarathnam, 2012). In brief, the samples were clustered by

constructing dendograms (Griffiths et al., 2000; Zhang and Fang, 2000; Kosman and

Leonard, 2005) using the Phylogeny Inference Package (PHYLIP, v 3.68) selecting the

unweighted pair group method with arithmetic mean (UPGMA) algorithm. The distance

matrix was developed using Pearson's correlation coefficient for difference in the

banding pattern between two samples and used as an input for the PHYLIP program. The

data were futher analyzed using principal component analysis (PCA) available in the

MATLAB (v.7.6, R2008a) software program.

Taxonomic units in major DGGE bands were identified by excising, cloning and

sequencing a number of clones from each major band as described elsewhere (Tale et al.,

2011; Navarathnam, 2012). In brief, bands used for PCA and cluster analysis were

excised and eluated from the DGGE gel in 50 µL of autoclaved water for 12 hr at 4 ^{0}C.
After elution, DNA fragments from each band were PCR-amplified for *mcrA* specific,
mcrF and *mcrR* primers as described above. The PCR-amplified products were cloned
using a cloning kit (TOPO TA Cloning® Kit , Invitrogen, Carlsbad, CA, USA) per the
manufacture's instructions. Nine white colonies containing plasmids with amplified
product were randomly selected and PCR-amplified with PUCF (5'-
GGAATTGTGAGCGGATAACA-3') and PUCR (5'-GGCGATTAAGTTGGGTAACG-
3') primers under the following PCR conditions: initial denaturation at 94°C (5 min),
annealing 30 cycles of 94°C (1min), 55°C (1 min), and 72°C (1 min), and a final
extension at 72°C (10 min). The presence and size of the PUC-amplified PCR products
was confirmed by running the agrose gel, and the products were cleaned using a clean-up
kit (Ultra Clean^{TM} PCR Clean-up^{TM} Kit, MoBio Laboratories, Carlsbad, CA, USA). The
purified PCR products were sequenced using a capillary automated DNA sequencer
(Applied Biosystems 3730XL, Foster City, CA, USA) at the DNA sequencing facility,
University of Chicago Cancer Research Center. The vector segments were removed from
cloned sequences and the remaining sequence was compared to GenBank® sequences
using the Basic Local Alignment Search Tool (BLAST).

5.2.4   BMP testing using freeze-dried inocula

 BMP testing was performed on various substrates, from simple sugars to relatively
complex organics, including glucose, non-fat dry milk (Roundy's Inc., Milwaukee, WI,
USA), thin stillage (Ethanol Processing Industry) and dry dog food (Nutro Products Inc.,
Franklin, TN, USA). BMP testing was conducted as described elsewhere (Owen et al.,
1979; Anagelidaki et al., 2007; Anagelidaki et al., 2009). In brief, FDMB was
reconstituted in basal nutrient medium containing L-cysteine hydrochloride (500 mg/L)

as a reducing agent (Coates et al., 1996). The 160-mL serum bottles were inoculated with 50 mL of FDMB having a VSS concentration of 3.5 g/L. Test bottles received 65 $\pm$10 mg COD or mg VS of substrate, whereas blanks were not fed substrate to correct for endogenous CH_4 production. All bottles were sparged with O_2 free gas, sealed and incubated at 35 ^{0}C and 150 rpm as discussed above. Biogas production was measured daily using water lubricated syringe and re-injected into the bottles. CH_4 content in accumulated biogas was measured at the end of the test using a GC. The net CH_4 production from the substrate was calculated by subtracting the total CH_4 produced in blanks from the test bottles. The BMP value of organic substrates was expressed in mL CH_4/g COD or VS added. Prior to substrate addition, all bottles were incubated under similar conditions and degassed for 2 to 3 days until the endogenous biogas production was minimal. A similar procedure was followed for non freeze-dried biomass. All tests were performed in triplicate.

5.2.5 Statistical analysis

Statistical analyses of results for methanogenic activity, BMP and bioaugmentation tests were performed using the one-sided Student's t-test with unequal population variance at a 95% confidence level.

5.3 Results and Discussion

5.3.1 Methanogenic activity of freeze-dried biomass

These results revealed significant methanogenic activity was preserved, equal to 65 $\pm$4.5% and 42 $\pm$ 10.4% for H_2 and acetate, respectively, compared to non-dried methanogenic biomass even without following strict anaerobic procedures (Figure 5.1). These results are supported by our previous study which estimated significant number of both *mcrA* gene and transcript copies for FDMB. As compared to similar, non-dried

biomass, the FDMB contained 53% and 63% of *mcrA* gene and transcript copies per gram of VSS respectively. Also in previous work, the clone library results based on the *mcrA* gene showed that the biomass contained mainly three methanogenic genera; *Methanospirillum*, *Methanoculleus* and *Methanosaeta,* and the abundance of *Methanospirillum* was highest, followed by *Methanosaeta* and *Methanoculleus* based on the qPCR analysis on *16S rRNA* genus-specific primers.
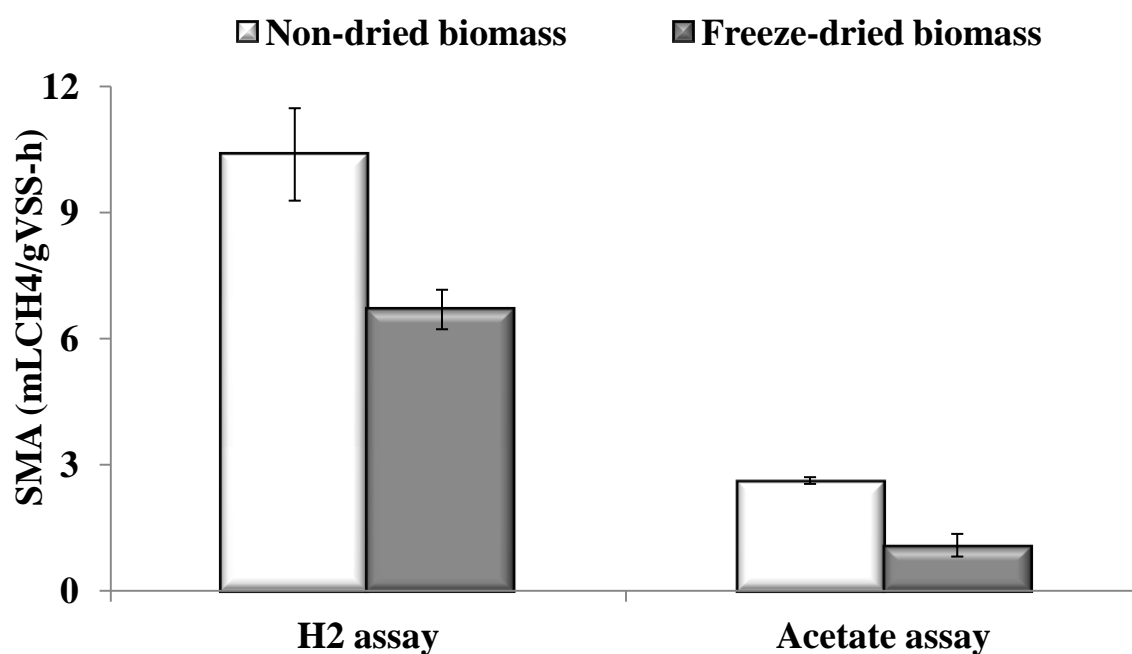
Figure 5.1. Specific methanogenic activity of freeze-dried methanogenic biomass before and after drying in the presence of air
Error bars represent the standard deviation of four replicates. Some error bars are small and not visible

5.3.2   Bioaugmentation effect of freeze-dried biomass

The bioaugmentation test was evaluated for 200 days, until transiently upset and control digesters achieved statistically similar performance (Figure 5.2).
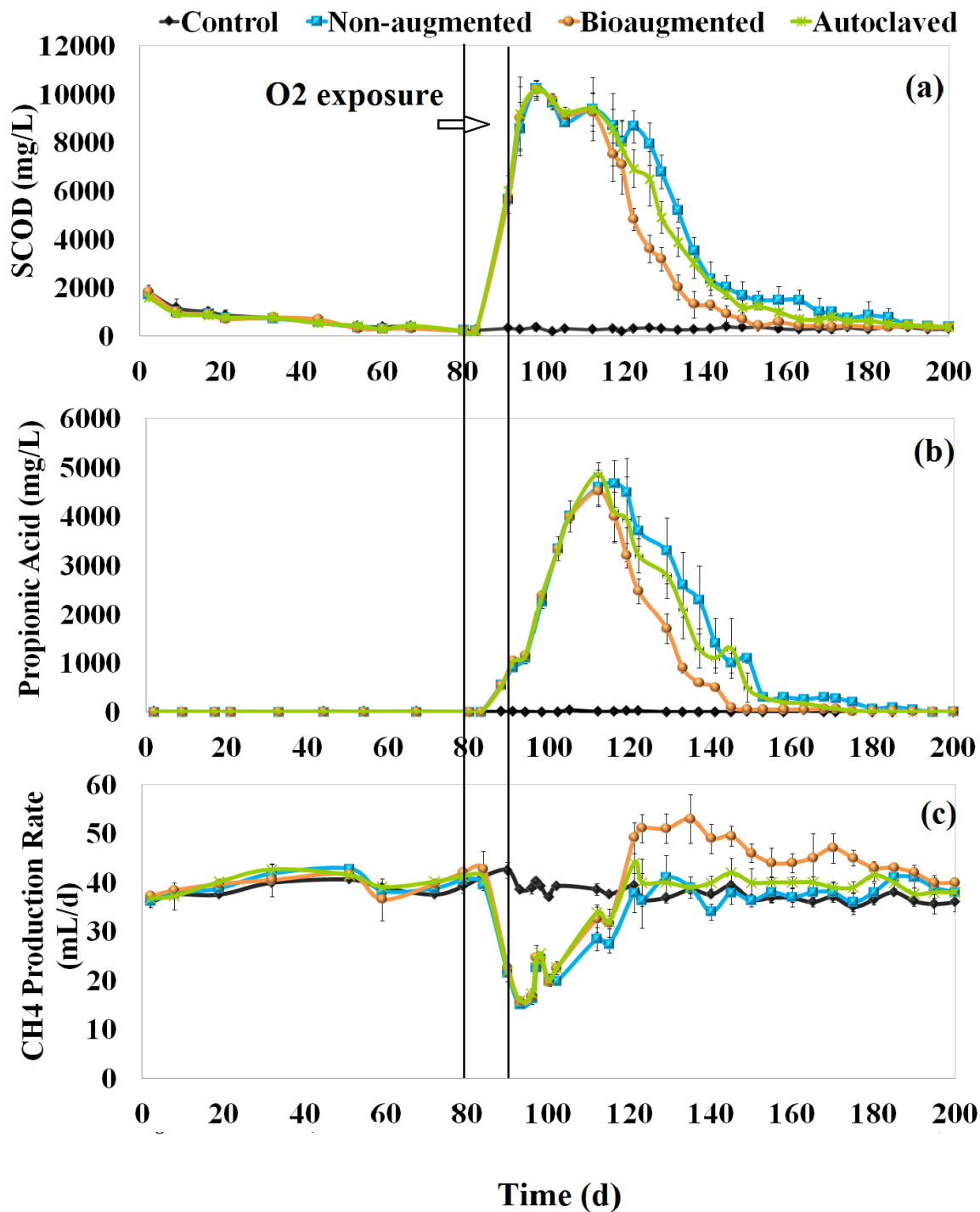
Figure 5.2. Bioaugmetation effect of freeze-dried methanogenic biomass on transiently upset anaerobic digesters
(a) SCOD (b) Propionic acid (c) CH$_4$ production rate
Error bars represent the standard deviation of three replicates. Some error bars are small and not visible.

5.3.2.1 Digester Monitoring

After seeding, the digesters required 60 days (3 SRTs) to reach quasi steady state, and then continued with statistically the same performance in terms of average effluent SCOD reduction (Figure 5.2a), average daily CH_4 production (Figure 5.2c), digester pH and VFA concentrations, over 80 days (4 SRTs) of operation until the digester was exposed to O_2. During this period, average effluent SCOD was 300 ±100 mg/L, average CH_4 production was 38±5 mL/d, average pH was 7.35 ±0.1 and all VFA concentrations were less than 15 ± 0.5 mg/L. The digesters were exposed to O_2 over a week from days 81 to 87, and the FDMB was used from day 90 to 150 (2 months) to bioaugment the transiently upset digesters.

Effluent SCOD: During and after oxic exposure, the average effluent SCOD from all upset digesters increased dramatically. The effect continued until day 98 when the highest average effluent SCOD of 10,000 ±400 mg/L was reached which was 33 times higher than the average SCOD of control digesters. The influence of bioaugmentation was seen after day 120, (approximately 1.5 SRTs after bioaugmentation initiated), in terms of statistically lower effluent SCOD in bioaugmented digesters compared to non-augmented and autoclaved digesters. The bioaugmented digesters attained statistically the same average effluent SCOD as the control digesters (300 ±100 mg/L) on day 153 which was 32 days sooner (approximately 1.5 SRT) than non-augmented and autoclaved digesters. During this period, the average SCOD removed by bioaugmented digesters was 24% ($p > 0.01$) and 17% ($p > 0.05$) higher compared to non-augmented and autoclaved digesters, respectively.

Effluent VFAs: Immediately after the toxic event, the VFA concentrations significantly increased. The maximum VFA concentrations in upset digesters was HAc (880 ±150 mg/L), HPr (4850 ±250 mg/L), Iso-HBu (574 ±17 mg/L), HBu (810 ±80 mg/L), Iso-HVa (580 ±50 mg/L), and HVa (20 ±5 mg/L). The HAc, HBu, HVa that accumulated in upset digesters quickly decreased in a period of 0.5 SRTs after the maximum built up. However, HPr, Iso-HBu and Iso-HVa lingered in the digesters and decreased more slowly. Compared to other organic acids, the accumulation of HPr concentration was higher in the upset digesters and decreased slowly during the recovery period (Figure 5.2b). The HPr degradation is thermodynamically challenging and its accumulation is often found in poorly performing or upset anaerobic digesters (Ahring et al., 1995; Pind et al., 2003; Mechichi and Sayadi, 2005; Schauer-Gimenez et al., 2010) due to imbalance in the harmony of propionate degrading bacteria and H_2-utilizing methanogenic archaea (McCarty and Smith, 1986). The typical range of HPr toxicity to inhibit CH_4 production is considered to be between 1000 to 2000 mg/L (Pullammanappallil et al., 2001; Dogan et al., 2005; Ma et al., 2009; Schauer-Gimenez et al., 2010). Wang et al., (2009) reported that HPr concentrations greater than 900 mg/L caused a decrease in CH_4 production rate and methanogenic microbial population in anaerobic digesters. Dogan et al., (2005) reported a decrease in CH_4 production of 50% at 3500 mg/L and 100% at 5000 mg/L of propionate. In contrast, no inhibition on CH_4 production was observed by others at HPr concentrations of 5000 to 6000 mg/L (Hajarnis and Ranade 1994; Gallert and Winter, 2008). The differences in the HPr tolerance level were attributed to the type of waste treated, digester configuration and operational parameters (Ma et al., 2009). It may also largely depend on the microbial community

structure in the system. Our results showed a decrease in CH_4 production when the HPr concentration increased to more than 1000 mg/L. It remained suppressed with increasing HPr concentration and began increasing with decreasing HPr concentration. The HPr decreased at a statistically higher rate in the bioaugmented digesters compared to non-augmented and autoclaved digesters after 1.5 SRTs. The bioaugmented digesters attained average HPr concentrations less than or equal to 100 mg/L on day 145 which was 1.5 SRTs before non-augmented and almost one SRT before autoclaved digesters. During this period, the average HPr concentration removed by the bioaugmented digesters was 32% ($p > 0.01$) and 26% ($p > 0.05$) higher compared to non-augmented and autoclaved digesters, respectively.

CH_4 production rate: Due to the toxic event, the average CH_4 concentration in biogas decreased from 61 ±3% to 35±2% in all upset digesters. The lowest CH_4 production was noted on day 96 which was 18 ±3 mL/d, less than one half of the control digesters. Daily CH_4 production started to recover after the toxic event. The CH_4 production rate was significantly higher for bioaugmented digesters from day 123 to 175 (approximate 2 SRTs) compared to non-augmented, autoclaved and control digesters. During this period, the average CH_4 production was statistically different and it was 16% ($p > 0.01$) and 11% ($p > 0.05$) higher than non-augmented and autoclaved digesters, respectively.

5.3.2.2 Microbial community analysis

Methanogenic community dynamics in bioaugmentation testing was analyzed using duplicate digesters from each set at different times: day 80 (quasi steady-state before oxic exposure), day 120 and day 140 (recovery period after oxic exposure) and day 190 (new

quasi steady-state after oxic exposure). DGGE banding patterns for the *mcrA* gene were generated for biomass samples from all sets of digesters at each time. Six distinct bands were obtained from DGGE analysis which had the highest contribution to banding pattern variability. Based on the variation in banding pattern intensity data, the biomass samples were clustered using UPGMA algorithm (Figure 5.3) and PCA by plotting the first two principal components on x- and y-axes which explained the maximum variation in densitometric data of banding patterns (Figure 5.4). Clustering of biomass samples resulting from the UPGMA method was also superimposed on PCA results.
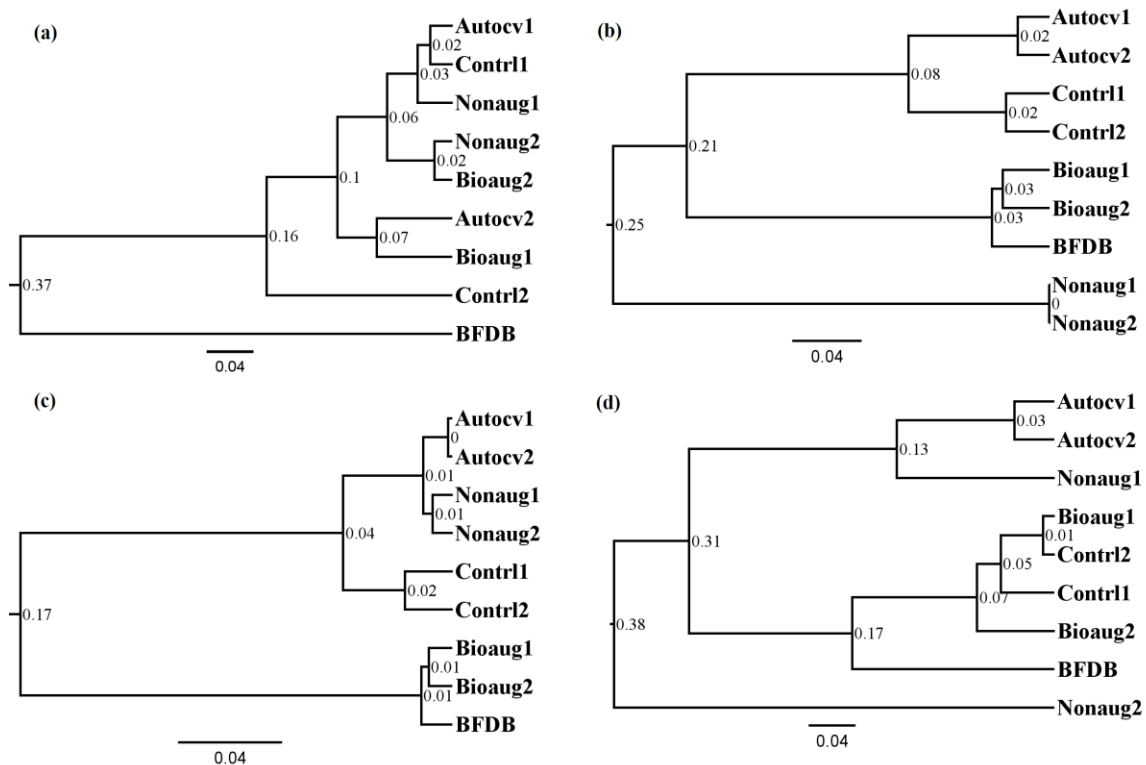
Figure 5.3. Cluster analysis of *mcrA* gene DGGE banding pattern using band intensity data

Day 80 represents quasi steady state before oxic exposure (a), Day 120 (b) and Day140 (c) both represent the recovery period of transiently upset digesters after oxic exposure, and Day 190 (d) represents a new quasi steady state after oxic exposure. The data were clustered based on the UPGMA algorithm. Biomass samples from each digester are named as follows: Contrl – Control; Nonaug – Non-augmented; Bioaug – Bioaugmented; Autocv – Autoclaved. The numbers 1 and 2 represent samples from duplicate digesters. BFDB – Bioaugmentation freeze-dried methanogenic biomass (the culture added to digesters).
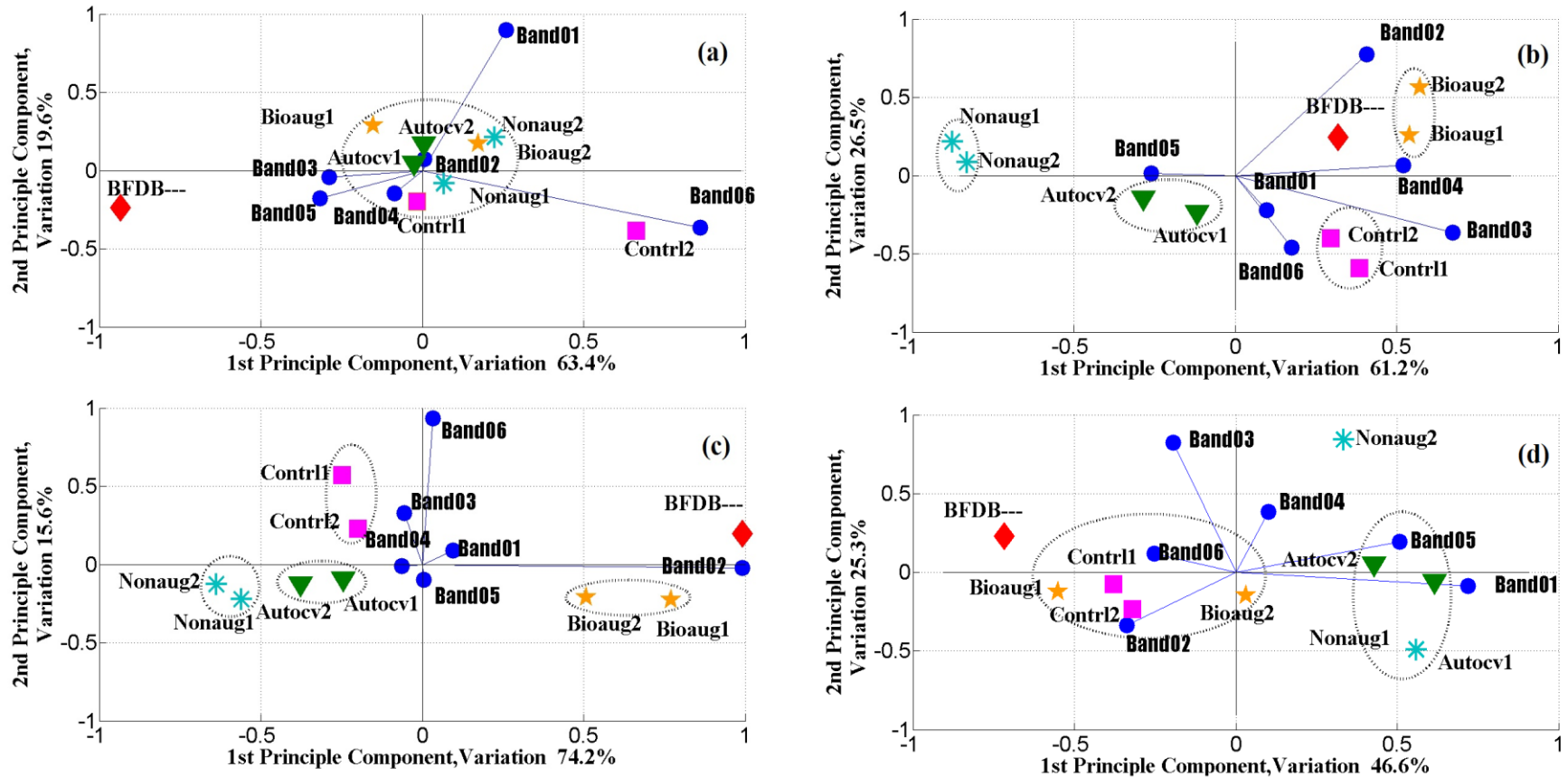
Figure 5.4. PCA analysis of *mcrA* gene DGGE banding pattern using band intensity data
Day 80 represents quasi steady state before oxic exposure (a), Day 120 (b) and Day140 (c) both represent the recovery period of transiently upset digesters after oxic exposure, and Day 190 day (d) represents a new quasi steady state after oxic exposure. The data were clustered based on the UPGMA algorithm. Biomass samples from each digester are named as follows: Contrl – Control; Nonaug – Non-augmented; Bioaug – Bioaugmented; Autocv – Autoclaved. The numbers 1 and 2 represent samples from duplicate digesters. BFDB – Bioaugmentation freeze-dried methanogenic biomass (the culture added to digesters).

The PCA results on day 80 (Figure 5.4a) showed that biomass samples from all sets of digesters were similar to each other, showing less variation on PCA coordinates and clustering in one major group except one sample from the control digester set. These results corresponded with steady state performance of digesters which were essentially the same on this day, indicating fairly similar methanogenic community structures in all digesters but a different FDMB community structure which was located at a distance from the major cluster on the PCA graph. Although the seed biomass and bioaugment FDMB was from the same source, differences in their community structures could be due to freeze drying of methanogenic biomass and changes in seed biomass development in 80 days due to operational variation.

The PCA results on day 120 and 140 (Figure 5.4b and 5.4c) showed that biomass samples from different sets of digesters were clustered differently and the samples from duplicate digesters were clustered together, indicating methanogenic community structures in different set of digesters were different and reproducible. These results correlated with different performance values of different sets of digesters. The bioaugmented digesters showed improved performance during these periods, and they clustered separately from non-augmented and autoclaved digesters. These results revealed that bioaugmentation caused a shift in the methanogenic community structure which was beneficial for the digester's improved perfomance. Similar finding was observed by Qu et al. (2005) where the bioaugmentation of *Sphingomonas xenophaga* culture significantly changed the microbial community dynamics and strongly persisted in the bioaugmented reactor for higher degradation of bromoamine acid, analyzed by ribosomal intergenic spacer analysis (RISA) technique. In this study, band 2 in

bioaugmented digester was more significant, suggesting that organisms represented by this band may have an important metabolic function for improved performance of transiently upset digesters, whereas bands 3 and 6 were more significant in steady state performance of the control digesters. PCA results on day 190 (Figure 5.4d) showed that biomass from non-augmented and autoclaved digesters clustered together, whereas biomass from bioaugmented digesters clustered with control digesters. These results suggest that although the performance of non-augmented and autoclaved digesters recovered in a manner similar to that of control digesters, they did not return to their original community structures. However, bioaugmentation using FDMB helped to re-establish upset digesters to their original methanogenic community structure that was similar to the controls; also, bands 2, 3 and 6 contributed to the control and bioaugmented digester communities.

The *mcrA* gene sequences in bands 1 through 6 were compared to GenBank® sequences (Figure 5.5).
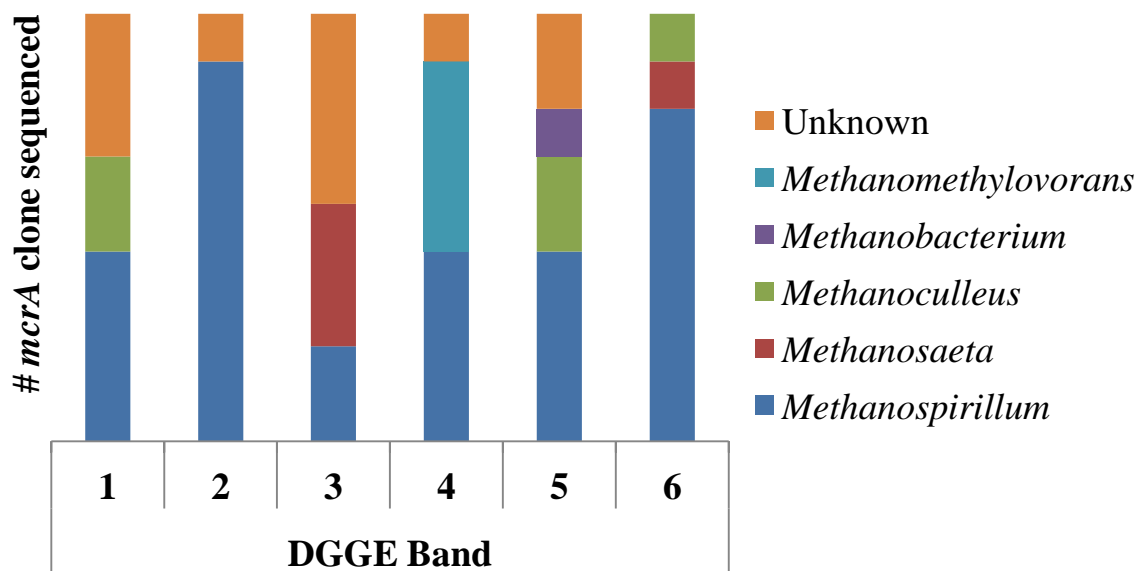
Figure 5.5. Methanogenic community analysis DGGE bands of *mcrA* gene
Nine clones from each band were analyzed for *mcrA* gene sequence and assigned to genera using ≥88% similarities with known reference sequences from GenBank[®]

Clones from a given band were similar to more than one methanogenic genus. Sequences related to *Methanospirillum* were found in all bands. This could be due to the dominance of *Methanospirillum* (84%) in the biomass, shown in the previously reported clone library and qPCR study by this author (see Chapter 2). In band 2, the presence of which was correlated to significantly improved performance of bioaugmented digesters, the majority of sequences were related to *Methanospirillum*. Whereas in bands 3 and 6, which contributed more during steady state periods of control digesters, the majority of sequences were related to *Methanosaeta* and unknowns, and M*ethanospirillum,* respectively.

The different species or strains of *Methanospirillum* ostensibly described by bands 2 and 6 relate to the difference in performance of the digesters. A previous study

discussed the positive influence of *mcrA* sequences related to *Methanospirillum hungatei* on higher specific methanogenic activity and rapid propionate utilization in some anaerobic cultures (Tale et al., 2011). Both *Methanospirillum* and *Methanosaeta* have been commonly found in anaerobic digesters (Laclerc et al., 2004; Steinberg and Ragan, 2008; Talbot et al., 2008; Lebuhn et al., 2009; Nelson et al., 2011) and are considered to be important digester community members for converting H_2 and acetate into CH_4 ,respectively.

5.3.3   BMP test using freeze-dried inoculum

FDMB and non-dried biomass were each used as inocula to perform BMP tests for different organic substrates, namely glucose, non-fat dry milk, thin stillage and dog food. No lag to initiate the biogas production was observed when non-dried inoculum was used for BMP tests, whereas a lag of 10 ±5 d was observed to initiate significant biogas production for FDMB BMP tests (Figure 5.6). However, the average BMP values of different substrates using FDMB inoculum were essentially identical to those obtained using non-dried biomass as inocula (.i.e, within 2.5±0.3%) (Figure 5.7).
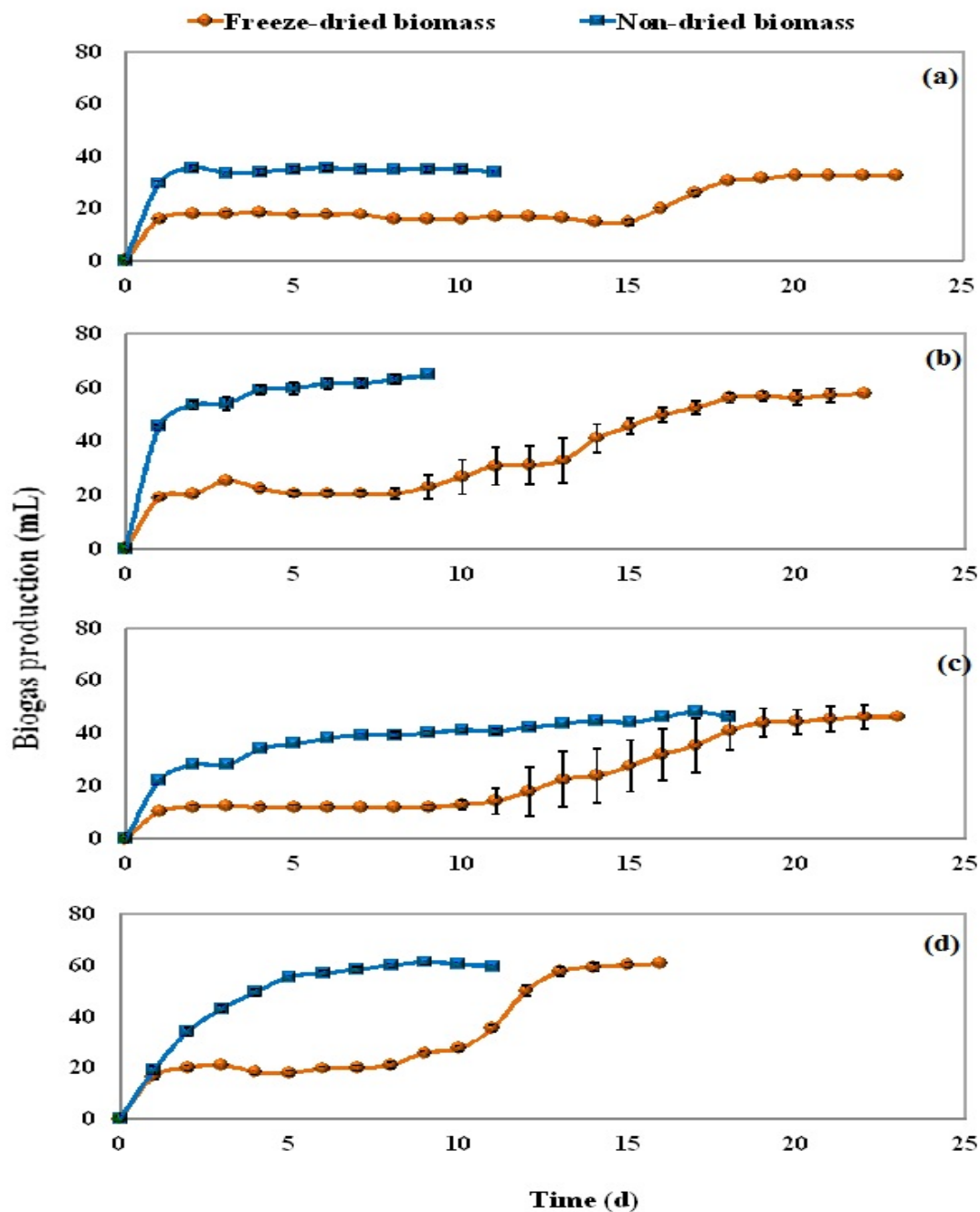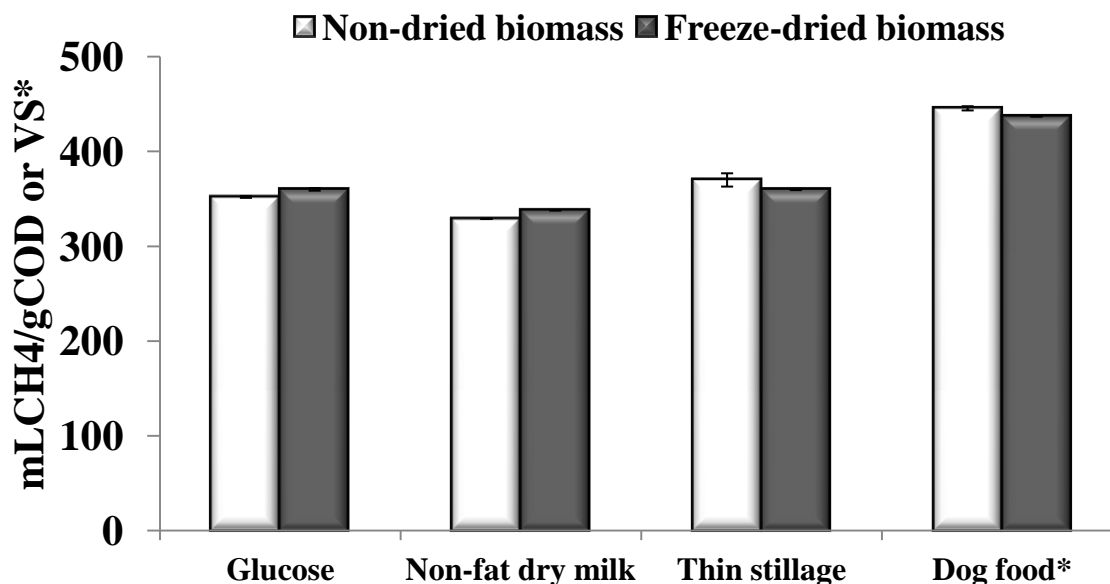
Figure 5.6. Average cumulative net biogas production from different organic substrates using freeze-dried and non-dried methanogenic inoculum
(a) Glucose, (b) Non-fat dry milk, (c) Thin Stilage, (d) Dog food
Error bars represents the standard deviation of three replicates. Some error bars are too small to visible

Figure 5.7. BMP values before and after freeze drying for different substrates
Error bars represent the standard deviation of 3 replicates. Some error bars are small and not visible
*BMP values for dog food calculated per gram of VS

5.4   Conclusion

The methanogenic cultures can be preserved by freeze drying. Significant methanogenic activity is maintained by methanogenic biomass after freeze drying even after handling and storage of the biomass under non-anaerobic conditions. The preserved methanogenic culture may be applied commercially since the biomass is stable, less expensive to ship and handle. It could be used as a source to encourage developing efficient customized methanogenic biomass for specific anaerobic biotechnology applications.

It is feasible to use FDMB as a bioaugment to improve the performance and stability of anaerobic digesters after toxic upset. Under the studied conditions, bioaugmentation using FDMB improved the performance and reduced the recovery time of transiently upset digesters due to O_2 exposure. However, in a similar study, others

found no benefit of bioaugmentation when H_2-utilizing methanogenic biomass used as a bioaugment for other digesters (Schauer-Gimenez et al., 2010). For no benefit, the adaptation of bioaugmenting biomass with seed biomass was anticipated.

In this study, methanogenic community analysis using DGGE demonstrated that bioaugmentation using FDMB shifted the methanogenic community which correlated to improved performance of digesters during recovery, and assisted to re-establish the original community structure after upset. DGGE of *mcrA* was found to be a useful finger-printing technique to analyze changes in methanogenic community structure over time in different digesters. Even though cloning and sequencing of DGGE bands showed sequences related to multiple methanogenic genera in a single excised band, the PCA analysis identified specific bands important to both digester recovery and steady state operation. Future research should focus on identifying genera or strains that are the most important taxonomic units in each band, perhaps by examining *mcrA* transcripts.

The FDMB can be used as inoculum for BMP testing. Using FDMB inoculum produced essentially the same BMP values as non-dried methanogenic biomass inoculum for simple and more complex substrates.

Future work remains to develop new beneficial cultures, optimized drying conditions and bioaugment dosing rates, and reduce the lag time in BMP testing.

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Chapter 6 Overall Conclusions

The preservation of methanogenic cultures is a potentially beneficial approach to develop efficient customized biomass for specific anaerobic biotechnology applications and may be commercially viable due to culture storage stability, less expensive shipping and handling, and easy availability and distribution.

The work herein successfully demonstrated the preservation and long-term storage of various customized mesophilic methanogenic cultures in the presence of air. The preservation of significant methanogenic functionality/activity after freeze and heat drying demonstrated the unique ability of mesophilic methanogens to tolerate the combined effect of drying, extreme temperature, and O_2/air. Especially notable was the survival of mesophillic methanogens to heat when the cultures were heat-dried at 104 ^{0}C for 12 hrs in air. These results challenge the conventional notion of methanogen sensitivity to O_2/air, extreme temperatures and drying, and indicate that some methanogens are resistant to harsh conditions, and their preservation is feasible. Our qPCR results revealed higher tolerance of *Methanospirillum* to these conditions than *Methanosaeta*, and *Methanoculleus*.

The freeze-dried cultures exhibited higher methanogenic activity immediately after drying than heat-dried cultures. However, the heat-dried cultures were more stable after drying and simulated long-term storage than freeze-dried cultures. These results are putatively due to the lower RMC values achieved after heat drying than after freeze-drying.

Different culture growth conditions also influenced the preservation and subsequent storage of methanogenic cultures. Hydrogenotrophic cultures developed with a small amount of glucose showed higher activity preservation after heat drying, whereas cultures developed with no glucose showed better preservation after freeze-drying. Furthermore, cultures developed in the presence of limited O_2 exhibited higher methanogenic activity after preservation and subsequent storage compared to cultures developed under strictly anaerobic conditions regardless of the preservation method employed. These results encourage future studies to develop a culture under different growth conditions to improve preservation and storage of methanogens. For instance, development of cultures frequently exposed to heat shock may improve activity after heat drying.

The effectiveness of glucose addition as a protective agent varied among the different preservation methods and the effectiveness was more profound for freeze drying than for heat drying. Future studies can be conducted to investigate the addition of sugar additives with higher T_g values which may provide higher activity after preservation and subsequent storage. Criteria for protectant selection on a commercial scale needs to be based on protectant availability, cost, and the excess COD exerted due to the additive when introduced in an anaerobic system.

The preserved methanogenic cultures could theoretically be applied commercially since the cultures are stable and less expensive to ship and handle. In this regard, the study proceeded to determine the feasibility of dried culture for bioaugmentation and standard laboratory tests. The bioaugmentation using a freeze-dried culture improved the performance after upset of anaerobic digesters. Under the studied conditions,

bioaugmentation increased the CH_4 production and reduced the recovery time of transiently upset digesters after O_2 exposure. Methanogenic community analysis demonstrated that bioaugmentation shifted the methanogenic community which correlated to improved performance of digesters during recovery and assisted in re-establishing the original community structure after upset. In the future, studies to, optimize the dose of bioaugmentation could be valuable to improve the performance and stability of anaerobic systems.

Using freeze-dried culture inoculum produced the same BMP values as produced using non-dried methanogenic biomass inoculum.

To the author's knowledge, this work was the initial study of methanogenic culture preservation and long-term storage in the presence of air. Future research remains to improve the methanogenic activity and/or to reduce activity lag of preserved cultures by optimizing 1) culture growth conditions, 2) process parameters of preservation methods, 3) storage conditions, and 4) protective agents.