Microwave-based Pretreatment, Pathogen Fate and Microbial Population in a Dairy Manure Treatment System

Ying Jin

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Zhiyou Wen

Katharine F Knowlton

Jactone Arogo Ogejo

Chenming (Mike) Zhang

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Abstract

Anaerobic digestion and struvite precipitation are two effective ways of treating dairy manure for recovering biogas and phosphorus. Anaerobic digestion of dairy manure is commonly limited by slow fiber degradation, while one of the limitations to struvite precipitation is the availability of orthophosphate. The aim of this work was to study the use of microwave-based thermochemical pretreatment to simultaneously enhance manure anaerobic digestibility (through fiber degradation) and struvite precipitation (through phosphorus solubilization). Microwave heating combined with different chemicals (NaOH, CaO, H_2SO_4, or HCl) enhanced solubilization of manure and degradation of glucan/xylan in dairy manure. However, sulfuric acid-based pretreatment resulted in a low anaerobic digestibility, probably due to the sulfur inhibition and side reactions. The pretreatments released 20-40% soluble phosphorus and 9-14% ammonium. However, CaO-based pretreatment resulted in lower orthophosphate releases and struvite precipitation efficiency as calcium reacts with phosphate to form calcium phosphate. Collectively, microwave heating combined with NaOH or HCl led to a high anaerobic digestibility and phosphorus recovery. Using these two chemicals, the performance of microwave- and conventional-heating in thermochemical pretreatment was further compared. The microwave heating resulted in a better performance in terms of COD solubilization, glucan/xylan reduction, phosphorus solubilization and anaerobic digestibility. Lastly, temperature and heating time used in microwave treatment were optimized. The optimal values

of temperature and heating time were 147^{o}C and 25.3 min for methane production, and 135^{o}C and 26 min for orthophosphate release, respectively.

Applying manure or slurry directly to the land can contribute to pathogen contamination of land, freshwater and groundwater. Thus it is important to study the fate of pathogens in diary manure anaerobic digestion systems. The goal of the project was to establish a molecular based quantitative method for pathogen identification and quantification, compare the molecular based method with culture based method and study pathogen fate in dairy manure and different anaerobic digesters. Result showed that molecular based method detected more *E.coli* than the culture based method with less variability. Thermophilic anaerobic digestion can achieve more than 95% pathogen removal rate while mesophilic anaerobic digester had increased *E.coli* number than fresh manure, indicating temperature is a key factor for pathogen removal.

In general, the overall goal of the study is to develop an integrated dairy manure treatment system. The microwave based pretreatment enhanced the subsequent biogas production and struvite precipitation, and the molecular tool based method provided a more precise and faster way to study the pathogen fate in various anaerobic digestions.

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Chapter 1 Introduction

In the USA, dairy operations with more than 1,000 cows meet the EPA definition of a CAFO (Concentrated Animal Feeding Operation), and are subject to EPA regulations. The current most common disposal way of dairy waste is land application. Risks associated with poorly managed land application of manure include greenhouse gas emissions, ecological system eutrophication, and groundwater contamination (Aitken et al., 2007; Effenberger et al., 2003). Applying animal manure on pasture and cropland as fertilizer may also increase the risk of pathogens contaminating water resources, entering the food chain, or infecting livestock. Therefore, proper management of waste produced by CAFOs has become a major environmental and human health concern.

Utilization of animal manure as a source of renewable energy and a valuable byproduct (such as agricultural fertilizer) has been a topic of interest in recent years. Among different conversion/utilization methods, anaerobic digestion is one of the most popular options. Anaerobic digestion is a series of processes in which microorganisms break down biodegradable material in the absence of oxygen. As shown in Figure 1.1, the processes begin with bacterial hydrolysis of the raw manure in which insoluble organic polymers such as carbohydrates or proteins are broken down into sugars or amino acids that are available for other bacteria consumption. Acidogenic bacteria then convert those sugars and amino acids into carbon dioxide, hydrogen, ammonia, and organic acids. Then, acetogenic bacteria will convert these organic acids into acetic acid, along with additional ammonia, hydrogen, and

carbon dioxide. Finally, methanogens convert acetic acid and hydrogen into methane and carbon dioxide.

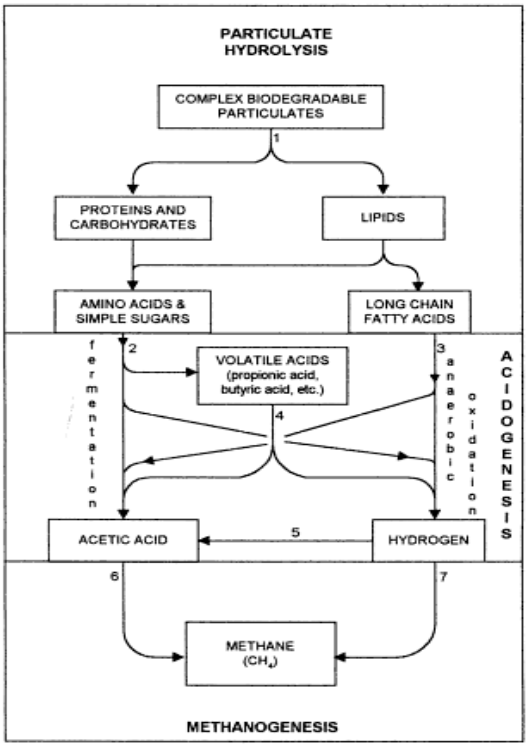


Figure 1.1 Steps involved in anaerobic digestion process (C.P. Leslie Grady et al, 1999)

Anaerobic digestion offers a number of benefits including odor control, reduction in greenhouse gas emissions, pathogen control, and beneficial products such as biogas (Demirer and Chen, 2005; Karim *et al.*, 2005; Uludag-Demirer *et al.*, 2005). However, the application of anaerobic digestion has been limited mainly due to two reasons. Firstly, animal waste has high content of fiber. These fibers that mainly consist of cellulose, hemicellulose and lignin are very difficult to digest. With mesophilic anaerobic digestion, the majority fiber components are unable to be degraded even after 20-30 days (Krause *et al.*, 2003; Lissens *et*

al., 2004b). The other limitation for anaerobic digestion is that most reactors are still operated as “black boxes”. The performance of digestion is optimized by parameters such as organic loading, hydraulic retention time, temperature and mode of mixing, but cellular level information at different operation conditions is rare.

Figure 1.2 illustrates our project objectives. To improve the digestion efficiency, a novel microwave-based thermochemical pretreatment method was employed to disrupt the “tough” structure of lignocellulose. Concurrently, organic phosphorus contained in the manure was converted into ortho-phosphorus, which can be further converted into struvite as slow release fertilizer. To better understand the microbial population dynamics of the digesters operated at different conditions, a molecular based method denaturing gradient gel electrophoresis (DGGE) was employed to identify and quantify the microbial communities. Finally, to evaluate the potential risk of disposal of anaerobic digestion effluent, the potential pathogens contained in the different anaerobic digestion streams were identified and quantified with a molecular based method.

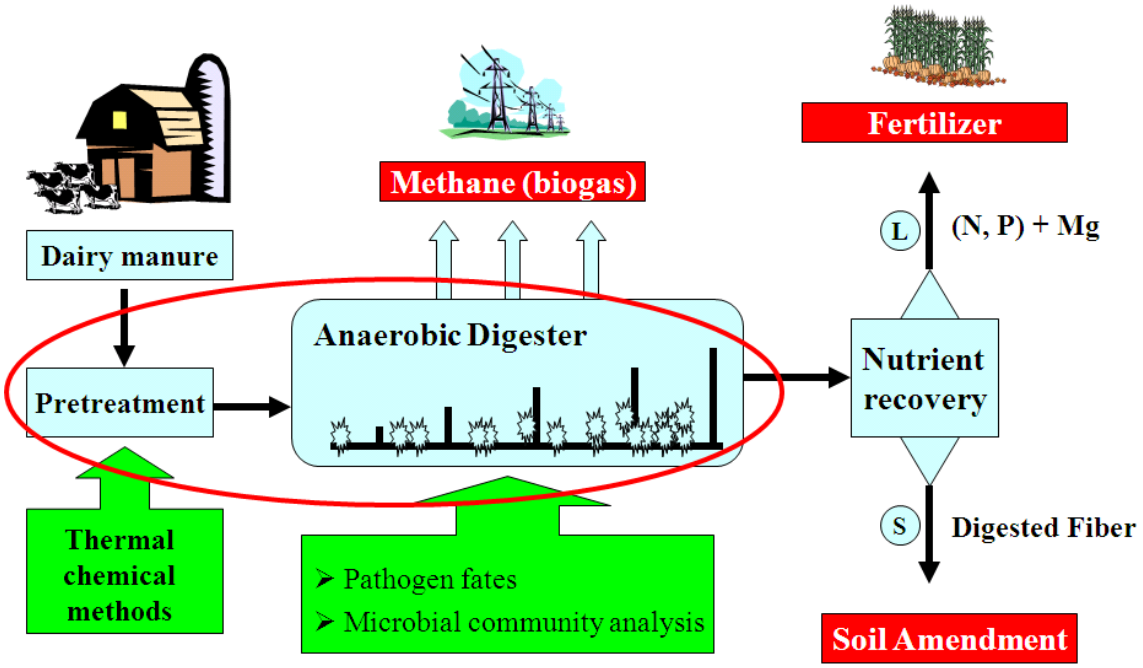


Figure 1.2 Scheme of project objectives

Chapter 2 Literature review

2.1 Pretreatment of anaerobic digestion

It is estimated that 160 million tons of manure (dry basis) are produced in the United States each year, with 75% are from dairy operations (Pathways and Monitoring in Natural and Engineered Systems). Currently, the use of manure is predominately done through land application, in which excessive amounts of manure may cause environmental problems. As a result, developing appropriate manure management practices has become a challenge to the U.S. agricultural industry.

Anaerobic digestion (AD) is an alternative for traditional manure management to alleviate environmental concerns and yielding profitable products such as biogas and fertilizer (Demirer and Chen, 2004; Demirer and Chen, 2005; Morris and Lathwell, 2004; Uludag-Demirer et al., 2005). With increasing energy prices, producing biogas by AD becomes more attractive to dairy farmers. In addition, AD has advantages in controlling odor and reducing greenhouse gas emissions.

Currently, the commercialization of dairy manure AD is limited by the slow rate of fiber degradation. The lignocellulosic fiber contained in dairy manure is very resistant to biodegradation due to its strong crystalline structure and other factors such as surface accessibility, pore size distribution, degree of polymerization, and lignin content(Liao et al., 2006; Lissens et al., 2004b). Thus, pretreatment before the anaerobic digestion is required to

disrupt the “tough” structure so that cellulose and hemicellulose can be easily degraded in the digesters.

Various chemicals have been used to pre-treat sludge before anaerobic digestion. Among these processes, the treatment using ozone and hydrogen peroxide is of special interest, because no salts are accumulated after pretreatment. The oxidation process hydrolyzes the large organic compounds in to smaller ones, and transfers non-biodegradable compounds into more easily degradable ones (Carballa et al., 2007; Muller, 2001; Rivero et al., 2006). Other oxidizers such as peroxymonosulphate (POMS) and dimethyldioxirane (DMDO) were also used to pre-treat wastewater sludge, resulting in more than two fold increase in biogas production (Dewil *et al.*, 2007). Acids or alkali were also applied in the pretreatment of activated sludge, municipal sludge, or animal manure. The usage of these chemicals was usually combined with other treatment such as thermal or ultrasound (Valo *et al.*, 2004; Vlyssides and Karlis, 2004).

Biological pre-treatment such as enzyme hydrolysis and codigestion are commonly performed. Enzyme hydrolysis is usually applied to oil- and grease-rich waste sludge because the enzymatic lysis can facilitate the cracking of big molecules into smaller ones by enzyme catalyzed reactions (Cammarota and Freire, 2006; Mendes *et al.*, 2006). Codigestion of organic wastes has also been applied for treating a mixture of several solid and liquid organic wastes. The bio-methane yield was increased because of the complementary nutrients from different sources. In addition, the equipment cost was reduced because of processing multiple waste streams in a single facility (Alatriste-Mondragon *et al.*, 2006; Paavola *et al.*, 2006).

Physical pre-treatment mainly includes ultrasound, electro-oxidation, and heating. The cell wall and the membrane of prokaryotes are composed of complex organic materials such as peptidoglycan, teichoic acids, and complex polysaccharides, which are not readily biodegradable. Ultrasound is a popular method for disintegration, the energy ruptures the cell wall and membrane and releases the intracellular organics into the bulk solution, which enhances the overall digestibility (Khanal et al., 2007; Watanabe et al., 2006). Electro-oxidation is an emerging technology; the principle of this technology is to decompose organic macromolecules into small molecules and consequently increase the biodegradability (Goncalves *et al.*, 2008; Song *et al.*, 2010).

Thermal based pre-treatment is the most widely used method in pretreating all kinds of waste materials. Generally, high temperature can be achieved by either conventional heating or microwave heating. Conventional heating has been used in earlier studies for treating activated sludge and cow manure (Bougrier et al., 2007; Yoneyama et al., 2006). In recently years, microwave heating has emerged as a promising new method due to its several advantages over conventional heating. Microwave heating is volumetric, and can selectively heat the more polar parts and create a "hot spot" within the heterogeneous manure slurry. For dairy manure which has high content of resistant fibers, this results in an "explosion" effect among the particles; and thus, greatly improve the disruption of the lignin "wrapper" encasing the cellulose and hemicellulose (Tokuyama and Nakamura, 2005). The fiber will be more easily degraded than with conventional heating. Thus, subsequent anaerobic digestion,

anaerobic bacteria can readily “attack” and convert those fibers into sugars. As a result, biogas yield will be significantly enhanced.

The combination of thermal treatment with chemicals has been widely studied for municipal waste (Bougrier et al., 2006b; Dereix et al., 2006; Eskicioglu et al., 2006; Pino-Jelcic et al., 2006). However, limited research has been performed on the pretreatment of dairy manure (Angelidaki and Ahring, 2000). Thus, in our research, a microwave-based thermochemical pretreatment was employed to investigate its effect on bio-methane yield and nutrient release.

2.2 Struvite precipitation

Dairy manure contains large quantity of phosphorus; land applying manure in excess of crop needs can cause ecological system eutrophication. Released phosphorus induces algal blooms which reduce light penetration and available oxygen in the water body. Algal blooms occurring near coral reefs can affect corals by preventing the penetration of sunlight, which is essential for the coral growth. Excessive phosphorus also weakens coral skeleton making corals more susceptible to physical damage (Shu *et al.*, 2006).

The most common phosphorus-removal technologies include constructed wetland systems, biological adsorption by various microorganisms, and metal precipitation (de-Bashan and Bashan, 2004). Constructed wetlands are a low-cost, low-tech process to control environmental pollution. They are confined system (as small as a bucket or as big as a large pond) planted with mainly aquatic, but sometimes with terrestrial plants. Inflow

wastewater current slowly flows either horizontally or vertically into one end and exits from the other end. The outflow is cleaner. Wetland removes phosphorus via plant consumption. However, since the system is not designed to remove nutrients, the phosphorus removal efficiency is low (de-Bashan and Bashan, 2004).

Enhanced biological phosphorus removal (EBPR) is the most important biological means for phosphorus removal from municipal wastewater. It is based on the selective enrichment of bacteria accumulating inorganic polyphosphate and involves microbial metabolic cycle via several microbial-accumulated biopolymers (polyphosphate, PHA, and glycogen). EBPR is a major avenue for contemporary and future development of phosphorus removal with microorganisms (de-Bashan and Bashan, 2004).

Another common approach for removing phosphate from wastewater is metal salt precipitation, which is an irreversible process (Donnert and Salecker, 1999a; Donnert and Salecker, 1999b). The most promising compound for recovery from wastewater plants is magnesium ammonium phosphate hexahydrate ($MgNH_4PO_4 \cdot 6H_2O$), commonly known as struvite, which precipitates spontaneously in some wastewater treatment processes (Stratful et al., 2001; Williams, 1999). Struvite has many uses with slow release fertilizer for agriculture being the most popular one (Gaterell *et al.*, 2000). It can also be used as a material in fire-resistant panels and in cement (Schuiling and Andrade, 1999). Additionally, if cheap production methods are developed, it could be used in other phosphorus-containing products such as detergents, cosmetics, and animal feed (de-Bashan and Bashan, 2004; Gaterell et al., 2000).

The formation of struvite is done through the following reaction (Uludag-Demirer *et al.*, 2005),

$$Mg^{2+} + NH_4^+ + PO_4^{3-} + 6H_2O \rightarrow MgNH_4PO_4 \cdot 6H_2O \qquad (1)$$

Much research has been conducted to study the formation of stuvite in various types of wastewater including anaerobic digestion effluent or dairy manure (Battistoni et al., 2001; Battistoni et al., 2002; Moerman et al., 2009; Qureshi et al., 2006). A model of stuvite formation in swine manure was developed by Celen et al (2007). The effectiveness of struvite formation is affected by pH, supersaturation ratio, temperature, mixing energy, present of foreign ions and the availability of orthophosphate (Le Corre *et al.*, 2009).

The pH is one of the main factors influencing the crystallization process, as it is linked to the notion of solubility and supersaturation. At a fixed pH, the supersaturation level of the solution affects the crystallization process in terms of induction time and influences the rate of struvite crystals formation (Le Corre et al., 2009).

Temperature has a lower impact on struvite precipitation than pH and supersaturation (Durrant et al., 1999), it can affect struvite solubility and crystal morphology. Mixing energy (or turbulence) can also influence the precipitation of struvite. For instance, CO_2 liberation can cause an increase of pH in the solution, thus favoring the occurrence of struvite crystals.

Foreign ions are also known to affect the growth rates of crystalline compounds due to blocking of sites where crystals could form (Jones, 2002). It is known that the presence of calcium or carbonates ions in the media affects negatively the growth rate and can lengthen the induction time (Koutsoukos et al., 2003).

In general, dairy manure contains more organic phosphorus and polyphosphate than orthophosphate; converting those organic phosphorus forms into the soluble orthophosphate form is crucial for successful struvite formation. Thus, in our research, a microwave based pretreatment method was developed to maximally release nutrients such as phosphorus and ammonia into bulk solution.

2.3 Characterization of microbe community in anaerobic digesters by denaturing gradient gel electrophoresis (DGGE)

Anaerobic digestion for treating animal manure for energy production is a promising technology because of odor control, reduction in greenhouse gas emission, and production of a beneficial by-product. Currently, most of the digestions are mainly regulated by physical and chemical parameters such as hydraulic retention time, organic loading and mixing modes. The performance of the process is usually evaluated by output parameters such as removal of chemical oxygen demand (COD), biological oxygen demand (BOD), total solid (TS), volatile solid (VS) and biogas production. Limited information is available for the microbial community dynamic during the anaerobic digestion processes. As shown in Figure 1.1, the anaerobic digestion is a mixed-culture process in which a consortium of organisms synergically acts to convert complex organic composite into biogas. Without microbial community population dynamic information, the operation of anaerobic digestion is like a "black box"; the effect of different operation parameters on the species and the quantities of archaea and bacteria is unknown. A better understanding of the distribution of microbial

communities and their quantity will provide a rational process control strategy for anaerobic digestion processes.

The traditional way of studying a microbial community is culture based method; the process usually includes the isolating individual cultures and the following characterization of their physiological, biochemical and morphological properties. However, such traditional methods have some limitations. First, due to the fastidious nature of the bacterium and the insufficient information of standard culture methods, in vitro cultivation on artificial grow medium are difficult for many organisms especially for those anaerobic archaea. Second, many organisms require syntrophic interactions with others, and thus cannot be cultured individually. Lastly, many organisms share similar physiological, biochemical, and morphological characteristics, and thus cannot easily be distinguished from each other (Chan, 2000; Zhang, 2001).

The 16s rRNA-based technology provides a reliable way to characterize microbial communities in anaerobic digesters. The 16s rRNA is a 1542 base pairs long component of the small prokaryotic ribosomal subunit. The sequence of 16s rRNA (or the corresponding gene, r-DNA) contains both the unique region which is specific for different microbial species, and the conserved region which is common for different species. In this sense, the gene sequences contain hypervariable regions which can provide species-specific signature sequences(Case *et al.*, 2007). This character makes the 16s ribosome RNA an ideal molecule inside the cells to identify and quantify the different microbial species among a mixed culture system.

Various molecular techniques have been developed to analyze 16S rDNA, including denaturing gradient gel electrophoresis (DGGE) for charactering a fraction of 16S rDNA; cloning and sequencing of full 16S rDNA; fluorescence in situ hybridization (FISH) with oligonucleotide probes; group-specific membrane hybridization; quantitative PCR; and terminal restriction fragment polymorphism (T-RFLP) (Chan, 2000; Zhang, 2001). To date, various mixed microbial systems have been investigated by 16S rDNA-based molecular techniques. For example, changes in the bacterial and archaeal species during the start-up of the cattle-manure anaerobic digestion process was characterized by their 16S rDNA sequences (Chackhiani *et al.*, 2004). The microbial community of the psychrophilic anaerobic digestion of wastewater was characterized by T-RFLP (McHugh *et al.*, 2004) and DGGE techniques (Connaughton *et al.*, 2006). Quantitative information of the anaerobic bacteria community was also evaluated by real-time PCR (Sawayama et al., 2006; Sylvester et al., 2004; Sylvester et al., 2005; Yu et al., 2005). Some microbial communities with special functions have also been investigated, including hydrogen-producing sludge (Fang *et al.*, 2002; Zhang *et al.*, 2002); sulfate-reducing bacteria (Menert et al., 2004; Thabet et al., 2004; Zhang and Fang, 2001); phosphate-removing activated sludge (Liu et al., 2005; Zhang et al., 2005a); denitrifying biofilm (Koenig *et al.*, 2005); and phenol-degrading sludge (Zhang et al., 2005b). With the cellular level information, researchers have a clearer understanding of the process.

Among the above methods, DGGE provides a simple and efficient method for microbial community characterization. The principle of DGGE is that the hydrogen bonds formed

between complimentary base pairs in GC rich regions ‘melt’ (strand separation or denaturation) at higher temperatures than regions that are AT rich. With DGGE, a small sample of DNA (or RNA) is applied to an electrophoresis gel that contains a denaturing agent; certain denaturing gels are capable of inducing DNA to melt at various stages. As a result of this melting, different DNA molecules can spread through the gel at different locations, and thus, can be separated and purified from gel for further analyses. When DNA separated by electrophoresis through a gradient of increasing chemical denaturant (usually formamide and urea), the double stranded DNA starts dissociating and the mobility of the molecule begins decreasing at certain concentration (Fig 2.1). So the branched structure of the single stranded moiety of the molecule becomes entangled in the gel matrix and no further movement occurs. Sequence differences in DNA fragments would cause them to partially melt at different positions in the gradient and therefore "stop" at different positions in the gel (Fischer and Lerman, 1980; Fischer and Lerman, 1983). By comparing the gel images of the polymorphic DNA fragments side by side, researchers can detect small differences in two samples or fragments of DNA. More precise information can be acquired by sequencing gel fragments.

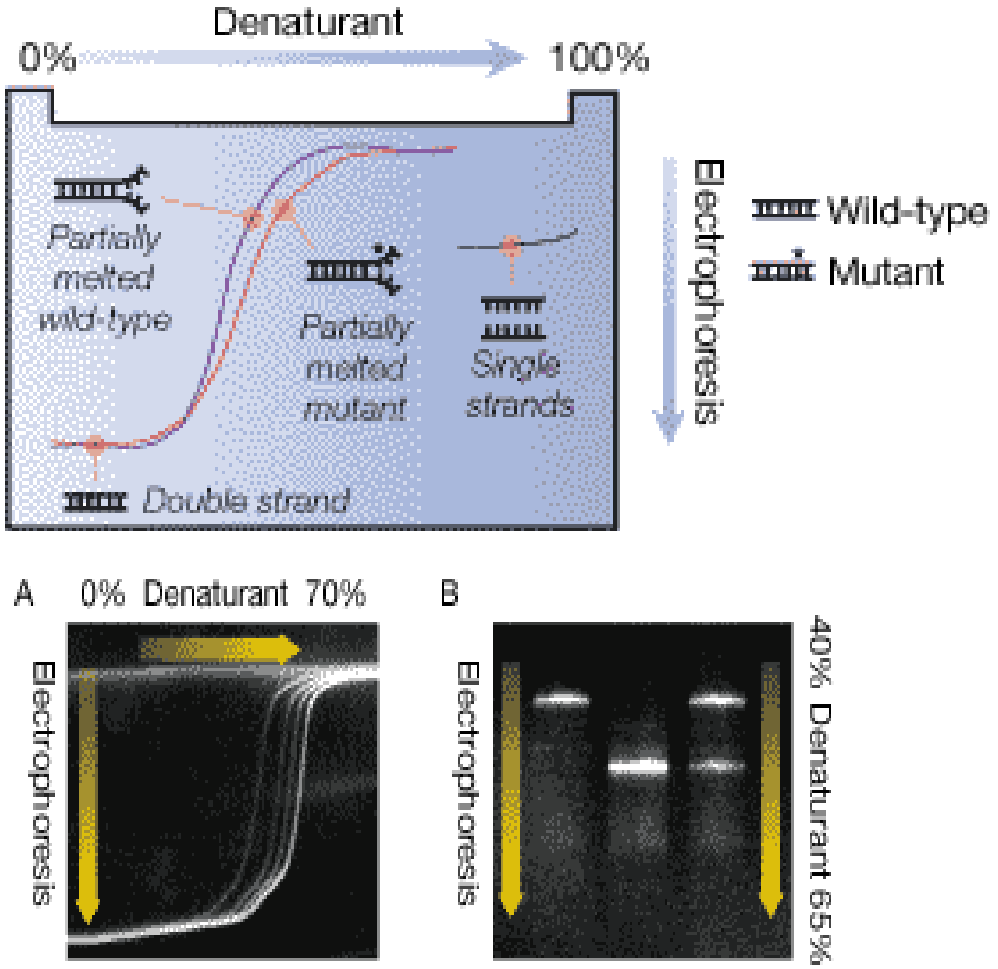


Figure 2.1 Illustration of Denaturing gradient gel electrophoresis (DGGE) principles (D-code universal mutation detection system, Bio-rad)

2.4 The fates of pathogens in dairy manure anaerobic digestion systems

Pathogen contamination of water resources is a risk to human health. This contamination originates from various sources such as wildlife, urban runoff, landfills, sewer overflow, and animal waste (Pathways and Monitoring in Natural and Engineered Systems, 2007). Understanding the sources, fate, and transport of pathogens in water and wastewater treatment systems and various watersheds is important to protect food safety, keep

sustainable natural resources and environment, and improve the quality of human life. Many species of bacteria or protozoa exist in dairy waste (Table 2.1).

Salmonella and *Campylobacter* are the most frequent agents of bacterial gastroenteritis (Westrell et al., 2009). *Salmonella* can enter a farm via carrier animals, or contaminated feed and water. Low numbers (15-100 colony forming units [CFU]) of salmonella in water may pose a public health risk (Jyoti *et al.*, 2010). *Campylobacter* species are widespread in the environment and are commonly found in surface water and biosolids (Sahlstrom *et al.*, 2004). About two million cases of human *campylobacteriosis* occur per year in the United States, of which most are sporadic and a few are due to waterborne transmission.

Escherichia coli is part of the normal flora of the gut, and can benefit its hosts by producing vitamin K2 (Bentley and Meganathan, 1982), or by preventing the establishment of pathogenic bacteria within the intestine (Reid *et al.*, 2001). However, certain strains of *E.coli* can cause disease in humans and animals. For example, *E.coli* O157:H7 has become the leading food- borne pathogen for human (Bicudo and Goyal, 2003). It can cause bloody diarrhea and sometimes more severe diseases such as hemorrhagic colitis, hemolytic uremic syndrome, or thrombocytopenic purpura (CDC 1997).

Table 2.1 Major pathogenic bacteria and protozoa in livestock excretions and manure (Bicudo and Goyal, 2003)

Microorganism	Survival condition	Infective dose	Major source of contamination	Disease incidence	Reference
Bacteria					
Salmonella	pH 4-8, temperature 8-45℃, survive for long periods in soil and water	High	Animal food, fish, shrimp, sauces and salad dressing	2-4 million cases in USA per year	(Jones, 1980) (Reilly, 1981) (Calvert *et al.*, 1998)
Escherichia coli	Can grow in adverse environment like low temperature and pH, and survive for long periods in soil and water.	High, but 0157:H7 is low	Animal food, unpasteurized juices and vegetables, untreated water	73,480 in USA per year	(Feng, 1995)
Campylobacter	Sensitive to environment	Low	Raw chicken, raw	Leading cause of	(Valcour *et al.*,

	stresses, cannot survive in dry environments		milk, untreated water	bacterial diarrhea in USA	2002) (Stanley *et al.*, 1998)
Yersinia	pH4-10, temperature 4-43, survive long in soil and water	unknown	Meat, oysters, fish, raw milk, soil and water	infrequently	(Deboer and Nouws, 1991) (Andersen *et al.*, 1991)
Protozoa					
Cryptosporidium	Waterborne oocysts relatively resistant to disinfectants, but decrease markedly as temperature increases	low	Contaminated water and vegetables	Prevalence of about 2% population in the USA	(Garber *et al.*, 1994)
Giardia	Are known to survive for	low	Contaminated water	Implicated in 25%	(Levine *et al.*, 1991)

extended periods of time	and vegetables	of the cases of gastro- intestinal disease in the USA

*Yersiniosi*s related illness has about 17000 cases per year in the USA, Most of the outbreaks were linked to contaminated water or seafood (Sharma *et al.*, 2003). The exact cause of food contamination is unknown, but poor sanitation during production and processing, improper food storage and handling may be contributory factors (Bicudo and Goyal, 2003).

The protozoa *Cryptosporidium* is known to be highly resistant to environmental stress. It has been detected in drinking water and waste water, and is considered an important water-borne contaminant (Xiao and Ryan, 2008).

The protozoa *Giardia* is also known to be resistant to environmental stress. It can survive for weeks to months in freshwater, and is more resistant to chlorine than enteric bacteria. The major sources of contamination are discharges of treated or untreated sewage, run-off or discharges of dairy manure, and wildlife (Girones *et al.*, 2010).

Present dairy waste management practices involving long- term storage in lagoons and spraying onto croplands as fertilizer (Ibekwe *et al.*, 2002). Because of illness of residents who lived near manure-applied land (Bicudo and Goyal, 2003), concern is increasing about the potential hazardous exposure created by the bio-solid and manure application, runoff from agricultural fields after periods of rainfall, and release during high-wind events (Higgins *et al.*, 2007). Indeed, several outbreaks of food-borne diseases have been traced to dairy farms or cattle farms (Aitken et al., 2007; Effenberger et al., 2003; Kudva et al., 1998). Therefore, it is important to control pathogens in dairy manure.

Table 2.2 lists the possible practices that could reduce the pathogen prevalence in the environment. Some of the means such as thermal treatment are quite commonly used for biosolids. Studies showed that temperature is the most important factor for influencing the fate of pathogenic bacteria. Temperatures over 70 ^{o}C could cause instantaneous death of most bacterial pathogenic strains (Castro and Tufenkji, 2007; Lang and Smith, 2008) . At low or high pH conditions, some of the heat resistance of bacteria can be reduced (Lang and Smith, 2008). However, due to the large volume of sludge needing to be processed, common thermal pretreatment is not effective due to low heat transmission rate, which in turn results in the reduced pathogen removal efficiency.

Table 2.2 Management options and their effect on pathogen removal (Bicudo and Goyal, 2003)

Management options		Application	Effect
Diet changes		Dairy manure	Reduction of acid resistant *E.coli* and *Salmonella*
Production systems		Dairy manure	Reduction of *Salmonella* in swine manure
Vegetative filter strips		Dairy manure	Reduction of bacterial indicator and *Crytposporidium* oocysts
Physical and	Thermal treatment	Biosolid or slurry	Reduction of viruses
chemical treatment	Chemical addition	Dairy manure	Reduction of bacterial indicator
	Ozonation	Biosolid	Reduction of bacterial indicator
	Electrolytic treatment	Cattle slurry	Reduction of bacterial indicator
Biological treatment	Anaerobic lagoon	Dairy manure	Reduction of bacterial and viral indicator
	Anaerobic digester	Dairy manure	Reduction of bacteria and viruses
	Thermophilic aerobic stabilization	Dairy manure	Reduction of bacteria and protozoa
	Sequencing batch activated sludge reactor (SBR)	Biosolid or animal manure	Reduction of bacterial indicator

Anaerobic digestion (AD) is another effective way for biosolid pathogen removal. Mesophilic AD (MAD) is the most common method of stabilizing bio-solid to achieve Class B sludge. However, the USA Environmental Protection Agency (EPA) is considering options for upgrading stabilization technology to produce Class A bio-solid (Viau and Peccia, 2009). Thermophilic anaerobic digestion (TAD) has much higher pathogen removal rate than MAD although high energy input will reduce the economic viability of this process. Temperature-phased anaerobic digestion (TPAD), a patented process developed at Iowa State University, is a two-stage anaerobic digestion system, which operates at high thermophilic temperatures (typically 55^{o}C) in the first stage and lower mesophilic temperatures (typically 37 ^{o}C) in the second stage. By combining the thermophilic and mesophilic digestion processes into one, TPAD offers the advantages of both while eliminating the problems associated with two systems when operated independently such as low pathogen removal efficiency, low bio-methane production and high energy input (Sung and Santha, 2003). In addition, some research revealed that TPAD systems could remove 1000- 10000 CFU ml^{-1} more bacteria than MAD (Santha *et al.*, 2006; Viau and Peccia, 2009).

2.5 Molecular based method for pathogen identification and quantification

The ability to accurately identify and quantify pathogens presented in environmental samples is crucial to assess health risks. The traditional methods for this purpose involve cultivation in appropriate culture media and subsequent biochemical or immunological characterization (Girones *et al.*, 2010). Culturing is a laborious and time-consuming procedure which usually requires one to several days to obtain results. For some zoonotic pathogen (pathogens that can be transmitted from animal to human) like *M. paratuberculosis*, it takes even longer (8-16 weeks)

and cultures are often lost due to the contamination with other microorganism (Grewal *et al.*, 2006). In some extreme cases, attempts to culture pathogen like *Helicobacter pylori* have been largely unsuccessful, and whether it can survive in environment especially in an infectious state is still debatable (Queralt *et al.*, 2005). In addition, the culture medium frequently lacks sufficient specificity, so false-positive results are found in many cases and a further confirmatory assay (such as immunoassays) is required (Aitken *et al.*, 2005). For many well-known and emerging pathogens, appropriate culture methods for environmental samples and biochemical schemes for valid species level identification are lacking (Dong *et al.*, 2008). In summary, due to the fastidious nature of bacteria and insufficient culture methods, very few quantitative methods have been developed (Dong et al., 2008; Girones et al., 2010; Percival and Thomas, 2009). This could be critical if decisions on require quickness and preciseness.

In some cases, concentrations of pathogen may be too low for culturing. Though the absolute number is still high enough to cause infection, with *salmonella*, the detection limit in waste samples by culturing method is as high as 10^7 CFU/g, but the infection dose to cause clinical illness can be as few as 800 organisms. Some researchers use one step enrichment method to reduce the detection limits. In this method, 1 g of waste samples is mixed with 9 ml of enrichment, and then the mixture is cultured at 37 ^{o}C or 43 ^{o}C for 24 hours. The enrichment broth varies based on the organism (Burtscher and Wuertz, 2003). Although the enrichment step reduced the detection limit by 3-4 log number CFU per g sample, it brings difficulties in precise quantification.

In other cases, bacteria in environment tend to attach to particles or fiber surface, and the attached growth would form biofilms (Leslie Grady et al, 1999). When cultured, the target bacteria might be embedded in biofilms and not be accessible to the standard techniques,

therefore causing the underestimation of pathogen numbers in samples.

Last but not the least, a pathogen that undergoes severe environmental stress such as nutrient deprivation, metals, chlorine, or low temperature could enter a state called "viable but non-culturable" (VBNC), and pathogen are undetectable at that state (Higgins *et al.*, 2007). VBNC distinguishes these cells that are "intact and alive" but cannot form colonies on or in conventional bacteriological media (Colwell, R. R. 2000). Approximately 60 species of food and waterborne bacteria have been reported to enter VBNC state at certain conditions, including *Escherichia coli* O157:H7, *Salmonella enterica, Listeria monocytogenes,* and *Shigella dysenteriae* (Oliver, J. D. 2005). With *E. coli* O157:H7 and *S. enteric*, 90% of VBNC cells retained viability as determined by a microscopic viability assay (Makino *et al.*, 2000; Reissbrodt *et al.*, 2002; Smith *et al.*, 2002). Under certain conditions, VBNC pathogen can be recovered and become virulent in their natural hosts (Dinu *et al.*, 2009).

Molecular techniques, usually DNA based methods, provide a highly sensitive, specific, fast and cost effective tool to identify and quantify the pathogens in various samples. These methods are typically designed to detect and quantify specific gene segments of certain pathogen genomes. The specificity of the targeted gene increases the detection accuracy. Also, the availability of in vitro amplification though PCR also increases the detection sensitivity (Signoretto and Canepari, 2008). To date, the molecular techniques have been more and more used in evaluating pathogen removal rate and microbial source-tracking (MST) in drinking water and wastewater treatment plants (Albinana-Gimenez et al., 2009; Field et al., 2003; Hundesa et al., 2006). Additionally, the development of multiplex PCR allows simultaneous detection of several target genes in a single assay, and thus, the detection of several species of pathogen at one time (Marcelino et al., 2006; Maynard et al., 2005; Rao et al., 2009).

The DNA-based methods for pathogen detection have been mostly conducted for water-based samples with a low solid content. For detecting pathogens from high solid content sample, Chen et al (2007) applied DNA based methods for municipal bio solid to track and enumerate *E.coli* that are undetectable by culture-base measurement. However, there have been few reports on using this method for detecting pathogens from dairy manure samples. Therefore, the development of a robust method to accurately assess pathogens in complex manure matrices is needed. Dairy manure is an extremely complex matrix of which may consist inhibitors such as humic acid and protein that could reduce DNA polymerase enzyme activity on PCR (Techer *et al.*, 2010). In addition, the efficiencies of DNA extraction and the consequent DNA yield may vary significantly depend on the methods used and the type of environmental samples.

The presence of inhibitors for PCR and the variation of DNA extraction efficiencies make pathogen quantification based on targeted gene extremely difficult and problematic. Research efforts have been attempted to overcome those limitations. For example, a known quantities of DNA was spiked with digested bio solid and served as an internal standard to monitor the DNA extraction and PCR reaction efficiency (Chen *et al.*, 2006). This method, however, is not applicable for quantifying pathogens in complex manure matrix because of the fact that the high content of fiber will block the reagents from cell lysis and entrap the DNA from extraction (Hu *et al.*, 2010). Indeed, mixing known quantity of DNA template with the solid may account for extraction efficiency, but the cell lysis efficiency will still be underestimated. In our research, a novel molecular based quantitative method was developed to account for detection efficiency including extraction efficiency, lysis efficiency, and other inhibition. Various types of dairy manure were analyzed based on this method to evaluate the pathogen fate in different manure treatment systems.

Chapter 3 Enhancing anaerobic digestibility and phosphorus recovery of dairy manure through microwave-based thermochemical pretreatment

3.1. Introduction

Dairy manure is a major waste in the U.S. agricultural industry. Currently, the disposal of manure is predominately done through land application. Risks associated with poorly managed land application of manure include greenhouse gas emissions, ecological system eutrophication, and groundwater contamination.

Anaerobic digestion is an alternative to traditional manure management to alleviate environmental concerns and yield profitable biogas. However, the high content of fiber contained in the manure limits the overall efficiency of anaerobic digestion because the degradation of recalcitrant fiber is very slow. Pretreatment of manure fiber to break down its complex structure is an effective way for enhancing anaerobic digestibility. To date, limited research has been performed on the pretreatment of dairy manure (Angelidaki and Ahring, 2000) although municipal sewage sludge has been well studied (Bougrier et al., 2006b; Eskicioglu et al., 2006; Eskicioglu et al., 2007; Valo et al., 2004). Thermochemical pretreatment is the main method used in these studies (Bougrier et al., 2006b; Dereix et al., 2006; Eskicioglu et al., 2006; Pino-Jelcic et al., 2006).

In addition to fiber, dairy manure also contains phosphorus, which causes ecological system eutrophication when excessive amount of manure is land-applied. Recovering phosphorus in the form of struvite is an effective way to reduce phosphorus discharge to ecological systems. Struvite (magnesium ammonium phosphate, $MgNH_4PO_4 \cdot 6H_2O$) is a crystalline solid which can

serve as slow releasing fertilizer due to its lower solubility. The formation of struvite is done through the following reaction (Uludag-Demirer *et al.*, 2005),

$$Mg^{2+} + NH_4^+ + PO_4^{3-} + 6H_2O \rightarrow MgNH_4PO_4 \cdot 6H_2O \qquad (1)$$

The effectiveness of the above reaction is affected by several factors, one of which is the availability of orthophosphate. In general, dairy manure contains organic phosphorus and polyphosphate; converting them into a soluble orthophosphate is crucial for successful struvite formation. A variety of pretreatment processes have been attempted to release phosphate from complex phosphorus of municipal sewage sludge (Kuroda et al., 2002; Liao et al., 2005; Lo et al., 2008; Wong et al., 2006b). Recently, microwave-assisted sulfuric acid and/or hydrogen peroxide pretreatment was also used to treat dairy manure with a high phosphorus release (Pan *et al.*, 2006; Qureshi *et al.*, 2008).

Although various pretreatment processes have been investigated for enhancing the anaerobic digestion or phosphorus release, there have been no studies developing a treatment method that can simultaneously enhance the efficiencies of these two processes. Indeed, depending on the temperature and different chemicals used, the performance of a pretreatment process in terms of anaerobic digestibility and phosphorus solubilization may vary widely. The objective of this work is to evaluate various thermochemical pretreatment for simultaneously enhancing anaerobic digestibility and phosphorus solubilization of dairy manure. We used microwave assisted heating in the pretreatment because this heating method has proved more efficient (less heating time and lower heating temperature) due to its volumetric and rapid nature (de la Hoz *et al.*, 2005).

3.2. Hypothesis

The hypothesis of this study is: first, microwave heating has advantage over conventional heating because microwave irradiation can disrupt the complex structure of activated sludge floc and release extracellular polymer substances, proteins, and sugars into a soluble phase. Second, microwave heating can both enhance the biogas production and nutrition recovery

3.3. Materials and methods

3.3.1. Raw materials and sample preparation

Dairy manure was collected from the Virginia Tech Dairy Center in Blacksburg, Virginia. The center includes one free-stall barns confining about 150 cows each. Manure produced at the barn is hydraulically flushed (four times a day) into a pit, and then separated into solid and liquid fractions. The solids are collected for composting; the liquid flows into a storage lagoon and is reused for flushing. For the current study, fresh manure on the concrete pads of the barns was used as the raw material. TS, VS, COD and other characteristics of the raw manure were determined by method described in section 3.2.6.

3.3.2. Pretreatment of manure slurry by microwave-based heating

3.3.2.1 Experimental set-up

Microwave-based thermo-chemical pretreatment was conducted in a Sharp/R-21 HT domestic microwave oven with a frequency of 2.45 GHz. The detailed description of the system setup was described previously (Hu and Wen, 2008). In brief, the top of the microwave oven was opened to provide a small hole (ca. 5mm in diameter). A fiber-optic temperature sensor (UMI, FISO technologies Inc, Quebec, Canada) was inserted through this hole into a sealed vessel. The

vessel was a CEM Advanced Composite Vessel (ACV) system (CEM Corporation, Matthews, NC) which consisted of a Teflon liner, a composite sleeve, a control cap with a vent tube and a thermo-well. Temperature inside the vessel was monitored through a temperature sensor.

Raw manure was diluted with distilled water to a solid content of 6.67% (w/w); the slurry was mixed with chemicals described in Table 3.1. The selection of chemicals was based on previous studies (Liao *et al.*, 2005; Wong *et al.*, 2006a). Ninety (90) ml of this mix was placed into the microwave vessel and treated at 120 ^{o}C for 30 min. The procedures for controlling temperature and heating time were the same as described previously (Hu and Wen, 2008).

When pretreatment was completed, the vessel was removed from the microwave oven and cooled at room temperature. The treated slurry (90 ml) was diluted with distilled water to 200 ml and centrifuged at 15,000 ×g through a Beckman centrifuge; the solids settled in the centrifuge tubes were analyzed for glucan and xylan content. The liquid phase from the centrifuge tubes was further filtered through 0.45 μm filter papers (APHA, 1995); the filtrate was collected for analyzing soluble COD, soluble phosphorus, orthophosphate, and ammonium.

Table 3.1. Chemicals at different loadings used in the microwave-based pretreatments.

Run	**Chemical used**	**Loading**	**H_2O_2 addition**
A-1	NaOH	0.00175 mol/g DM	-
A-2	NaOH	0.00088 mol/g DM	-
A-3	NaOH	0.00088 mol/g DM	1% H_2O_2 (V/V)
B-1	CaO	0.0012 mol /g DM	-
B-2	CaO	0.0006 mol /g DM	-
B-3	CaO	0.0006 mol /g DM	1% H_2O_2 (V/V)

C-1	H_2SO_4	2% (V/V)[a]	-
C-2	H_2SO_4	0.5% (V/V)	-
C-3	H_2SO_4	0.5% (V/V)	1% H_2O_2 (V/V)
D-1	HCl	0.74% (V/V)[b]	-
D-2	HCl	0.185% (V/V)	-
D-3	HCl	0.185% (V/V)	1% H_2O_2 (V/V)
MW[c]	-	-	-

[a] 2% (V/V) means there are 2 ml H_2SO_4 in 100 ml slurry
[b] 0.74% (V/V) means there are 2ml HCl in 100 ml slurry
[C] MW refers to microwave treatment only, no chemical was added

3.3.2.2 Experimental design to optimize heating temperature and time

A central composite design was used to optimize temperature (T) and heating time (t) used in the microwave pretreatment. As shown in Table 3.2, the design matrix contained a 2^2 factorial design matrix with four axial points and three central points. The responses of runs 1-8 were the means of three replicates; the central point was run in triplicates (runs 9-11). Methane production from Biochemical Methane Potential (BMP) test and concentration of orthophosphate released from treated manure were used as responses. These were written as functions of variables, i.e.,

$$Y = a_0 + a_1 \cdot X_1 + a_2 \cdot X_2 + a_{11} \cdot X_1^2 + a_{22} \cdot X_2^2 + a_{12} \cdot X_1 \cdot X_2 \qquad (2)$$

where Y is the response (either methane production or orthophosphate concentration), X_1 and X_2 are the coded levels of the two variables (T and t), and $a_0, a_1, a_2, a_{11}, a_{22}, a_{12}$ are coefficients. The response and variables (in coded unit) were analyzed by the Response Surface Analysis function of the JMP software (SAS Institute Inc. Cary, NC) to obtain the values of the coefficients of Eq. (2).

Table 3.2. Central composite design of temperature (T) and time (t) used in microwave pretreatment

Run	Variables			
	T		t	
	Coded unit	True value	Coded unit	True value
1	+1	150^{o}C	+1	30 min
2	+1	150^{o}C	-1	10 min
3	-1	90^{o}C	+1	30 min
4	-1	90^{o}C	-1	10 min
5	0	120^{o}C	+1.41	34 min
6	0	120^{o}C	-1.41	6 min
7	+1.41	162^{o}C	0	20 min
8	-1.41	78^{o}C	0	20 min
9	0	120^{o}C	0	20 min
10	0	120^{o}C	0	20 min
11	0	120^{o}C	0	20 min

3.3.3. Pretreatment of manure slurry by conventional heating

Dairy manure slurry contained in a beaker was placed in an autoclave for conventional heating treatment. The solid content of the manure slurry, chemical loadings, heating temperature, and heating time were the same as those used in microwave-based pretreatment. The solubilization and biogas production of the treated manure slurry was determined and used as control to evaluate the performance of microwave-based heating.

3.3.4. Biochemical methane potential (BMP) test

Pretreated manure slurry (90 ml) as described in Section 3.2.2.1 was neutralized and mixed with 20 ml anaerobic seed and diluted to 200 ml with distilled water. The seed was a mix (1:1 ratio, v/v) of dairy lagoon sludge and rumen fluid which was found to be more productive than lagoon or rumen fluid alone (data not shown). The slurry was placed in serum bottles and flushed with N_2 gas for 1-2 min to create anaerobic conditions. The bottles were incubated in an orbital shaker at 37^{o}C with continuous shaking (170 rpm). The biogas (CO_2+CH_4) was measured by water displacement method, while methane was estimated by mixing the biogas with 1N NaOH solution and recording the gas volume after CO_2 absorption. Methane content was also calibrated with a Shimadzu 2014 gas chromatograph equipped with a thermal conductivity detector. The final methane production of the manure sample was corrected for the amount of methane produced from the seed.

3.3.5. Struvite precipitation

The pretreated manure slurry was centrifuged and filtered through 0.45 μm filter papers. The filtrate was placed in 100 ml beakers and $MgCl_2$ was added. As ammonium concentration well exceeded orthophosphate concentration in the solution, the amount of $MgCl_2$ addition was based on the amount of orthophosphate with 2:1 (mol/mol) of Mg:P ratio as the target. The solution was adjusted to pH 9.0, and continuously mixed with a magnetic stirrer bar at 200 rpm for 20 min. Then, the samples were centrifuged at 15,000 ×g at 4℃ for 20 min, and the supernatant was collected for analyzing residual orthophosphate concentration. The precipitate solid was freeze-dried at -20℃ by a freeze dry system (VWR) and prepared for SEM (Scanning Electron Microscope) imaging.

3.3.6. Analyses

Dry matter (DM), volatile solid (VS), total COD (TCOD), soluble COD (SCOD), ammonium, total Kjeldahl nitrogen (TKN), soluble phosphorus (soluble-P) and orthophosphate (ortho-P) were determined according to standard methods (APHA, 1995). Glucan and xylan content of the manure solid were analyzed according to NREL laboratory analytical procedures (LAP) 014 and 002 (Ruiz and Ehrman, 1996a; Ruiz and Ehrman, 1996b). The structures of precipitated struvite were observed with a Zeiss EVO 40 Scanning Electron Microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) as described previously (Hu and Wen, 2008).

3.3.7 Statistical analysis

The mean values and standard variation of the experimental results was calculated by Microsoft excel 2003. Statistical significance of the experimental data was tested using one way analysis of variance (ANOVA) by JMP. Tukey's pairwise comparisons were used to test differences between treatments.

3.4. Results

3.4.1 Manure characterization

Raw dairy manure contained 14.09% of dry matter (Table 3.3). The total COD is 820mg/gDM, and the soluble COD is 40.6 mg/g DM. The TP, soluble-P and otho-P are 5.7mg P/g DM, 1.13 mg P/g DM and 0.48 mg P/gDM, respectively.

Table 3.3. Characteristics of raw manure slurry [a]

Parameters	Values
Dry matter (DM, %)	14.09 ± 0.17

Volatile solid (VS, %)	12.29 ± 0.05
Glucan and xylan content (g/g DM)	0.37 ± 0.01
Total COD (TCOD, mg/L)	7741 ± 193.5
Soluble COD (SCOD, mg/L)	383.3 ± 7.6
SCOD/TCOD (%)	4.95
Total phosphorus (TP, mg P/L)	53.81 ± 0.38
Soluble phosphorus (Soluble-P, mg P/L)	10.67 ± 0.19
Orthophosphate (Ortho-P, mg P/L)	4.53 ± 0.09
Total Kjeldahl nitrogen (TKN, mg N/L)	278.02 ± 4,72
Ammonium (mg N/L)	50.69 ± 0.19

[a] Data are means of three replicates ± standard deviations.

3.4.2. Solubilization of manure components

The effectiveness of microwave-based pretreatment was first evaluated by the solubilization of various manure components. Fig. 3.1 shows the SCOD/TCOD ratio of the pretreated manure. All pretreated manure increased the SCOD/TCOD ratio (Fig. 3.1) as compared to raw manure (Table 3.3). Acid pretreatment, particularly at high loadings, resulted in a much higher COD solubilization (Table 3.1 and Fig. 3.1). In the acid pretreatment, adding H_2O_2 increased the SCOD/TCOD ratio significantly ($P < 0.05$) (C-3 vs. C-2; D-3 vs. D-2, Fig. 3.1). However, adding H_2O_2 to the alkaline pretreatment did not cause a significant ($P > 0.1$) difference compared with H_2O_2-free treatment (i.e., A-3 vs. A-2; B-3 vs. B-2, Fig. 3.1).

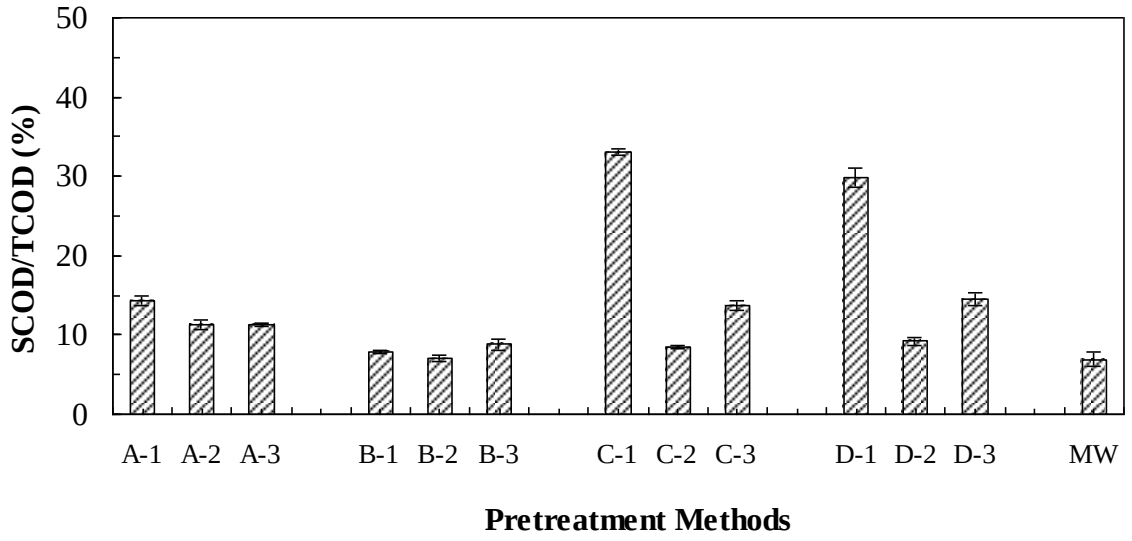


Fig. 3.1. SCOD/TCOD ratios of manure samples treated by different methods as described in Table 2. (A-1) manure treated with high loading of NaOH, (A-2) manure treated with low loading of NaOH, (A-3) manure treated with low loading of NaOH and H_2O_2; (B-1) manure treated with high loading of CaO, (B-2) manure treated with low loading of CaO, (B-3) manure treated with low loading of CaO and H_2O_2; (C-1) manure treated with high loading of H_2SO_4, (C-2) manure treated with low loading of H_2SO_4, (C-3) manure treated with low loading of H_2SO_4 and H_2O_2; (D-1) manure treated with high loading of HCl, (D-2) manure treated with low loading of HCl, (D-3) manure treated with low loading of HCl and H_2O_2.

[a]Data are means of three replicates and error bars show standard deviations.

[b]Tukey's pairwise comparisons was performed at p= 0.05. A-2 vs A-3, B-2 vs B-3 are not significantly different; C-1 vs C-2 vs C-3, and D-1 vs D-2 vs D-3 are significantly different.

The microwave-based thermochemical treatment caused solubilization of glucan and xylan contained in the manure. As shown in Fig. 3.2, pretreatment with high loading of H_2SO_4 (C-1) resulted in the highest glucan and xylan reduction ($p<0.05$), followed by high loading of HCl

($p<0.05$) (D-1). For other pretreatment conditions, the remaining glucan and xylan after treatment was 75 - 95% of that in the original raw manure samples.

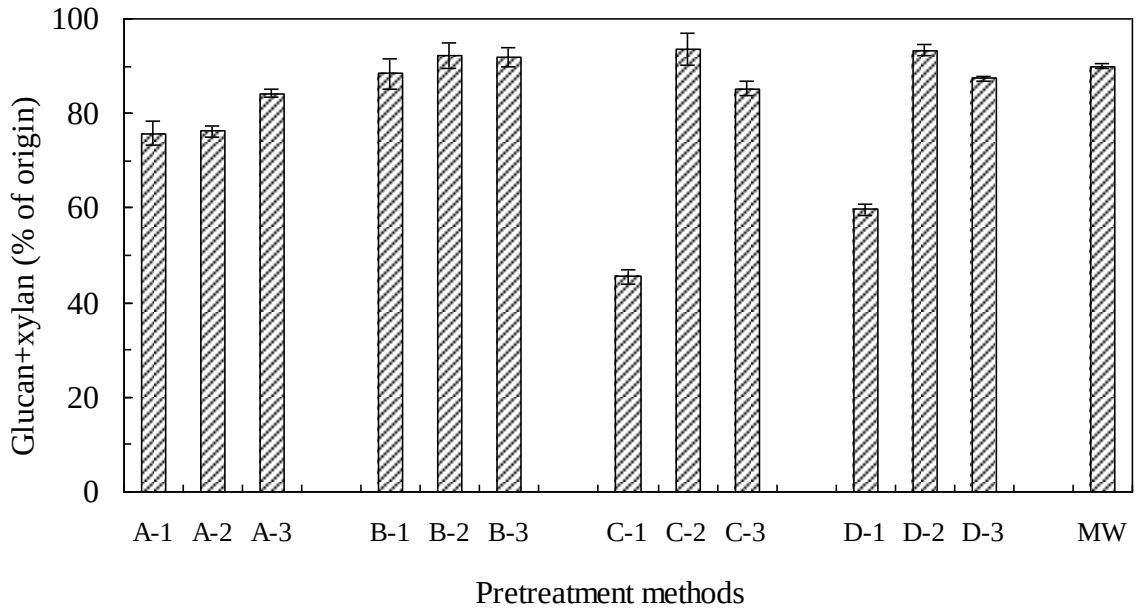


Fig. 3.2. Glucan and xylan content (% of the amount in the original raw manure sample) of manure treated by different methods. (A-1) manure treated with high loading of NaOH, (A-2) manure treated with low loading of NaOH, (A-3) manure treated with low loading of NaOH and H_2O_2; (B-1) manure treated with high loading of CaO, (B-2) manure treated with low loading of CaO, (B-3) manure treated with low loading of CaO and H_2O_2; (C-1) manure treated with high loading of H_2SO_4, (C-2) manure treated with low loading of H_2SO_4, (C-3) manure treated with low loading of H_2SO_4 and H_2O_2; (D-1) manure treated with high loading of HCl, (D-2) manure treated with low loading of HCl, (D-3) manure treated with low loading of HCl and H_2O_2.

[a]Data are means of three replicates and error bars show standard deviations.

[b]Tukey's pairwise comparisons was performed at $p= 0.05$. C-1 is significantly different with C-2 and C-3; D-1 is significantly different with D-2 and D-3; A-1 is not significantly different with A-2 and A-3; B-1 is not significantly different with B-2 and B-3;

Table 3.4 shows the concentrations of soluble-P, ortho-P, and ammonium of treated manure. All the pretreatments released soluble-P, ortho-P, and ammonium, the release efficiencies were dependent on the pretreatment methods. For example, CaO-based pretreatment (B1, B2 and B3) resulted in a lower release of soluble-P and ortho-P compared to other chemicals used; manure treated with microwave heating (MW) had less ammonium release than the combinations of microwave heating and chemicals.

Table 3.4. Soluble-P, Ortho-P, and ammonium content and release percentage of dairy manure treated by different methods described in Table 2 [a, b]

Run	Soluble-P		Ortho-P		Ammonium	
	mg P/g DM	Released (%)	mg P/g DM	Released (%)	mg N/g DM	Released (%)
A-1	3.30±0.06	38.09	1.96±0.16	25.96	8.29±0.22	9.92
A-2	3.13±0.02	35.04	1.84±0.10	23.88	8.10±1.44	9.28
A-3	2.81±0.02	29.47	1.89±0.03	22.73	8.26±1.12	9.83
B-1	2.65±0.02	26.68	0.76±0.06	4.95	8.52±0.72	10.70
B-2	2.29±0.02	20.28	0.70±0.03	3.82	8.04±0.72	9.05
B-3	2.43±0.02	22.77	0.81±0.03	5.74	8.76±0.86	11.50
C-1	2.92±0.06	31.42	2.21±0.10	30.36	9.49±1.01	14.01
C-2	2.79±0.03	29.19	1.92±0.10	25.26	8.89±0.86	11.97
C-3	3.10±0.11	34.47	2.02±0.13	27.19	9.05±0.28	12.49
D-1	2.68±0.05	27.23	1.30±0.06	14.42	9.37±1.14	13.61
D-2	2.78±0.30	28.91	1.23±0.13	13.21	8.64±1.19	11.09

D-3	2.62±0.08	26.12	1.27±0.10	13.98	8.66±0.29	11.29
MW	2.73±0.05	28.23	1.21±0.05	12.91	6.16±0.74	2.68

[a] Data are means of three replicates ± standard deviations.
[b] (A-1) manure treated with high loading of NaOH, (A-2) manure treated with low loading of NaOH, (A-3) manure treated with low loading of NaOH and H_2O_2; (B-1) manure treated with high loading of CaO, (B-2) manure treated with low loading of CaO, (B-3) manure treated with low loading of CaO and H_2O_2; (C-1) manure treated with high loading of H_2SO_4, (C-2) manure treated with low loading of H_2SO_4, (C-3) manure treated with low loading of H_2SO_4 and H_2O_2; (D-1) manure treated with high loading of HCl, (D-2) manure treated with low loading of HCl, (D-3) manure treated with low loading of HCl and H_2O_2.

3.4.3. Anaerobic digestibility

Anaerobic digestibility of pretreated manure was evaluated by the biochemical methane potential (BMP) test. Figs. 3.3A-3.3D show cumulative methane production of manures treated by different chemicals, and of raw manure and microwave-heated manure. These two types of manures generated a similar volume of methane over the whole incubation period (42 days) although microwave-heated manure generated more methane than raw manure for the first 25 days (Fig. 3.3).

As shown in Figs. 3.3A and 3.3B, the alkaline (NaOH and CaO) treated manures had higher methane production levels than raw manure and microwave heated manure; the improvement is independent of chemicals loadings and H_2O_2 addition. When H_2SO_4 was used to treat manure (Fig. 3.3C), however, the methane production was significantly (P<0.1) lower than alkaline-treated manure. The methane production at high H_2SO_4 loading was even lower than the raw manure (C-1, Fig. 3.3C). For manures treated with HCl, the methane production was better

than sulfuric acid treated manure (Figs. 3.3C and3. 3D), although high HCl loading decreased methane production to some extent (D-1, Fig.3.3D).

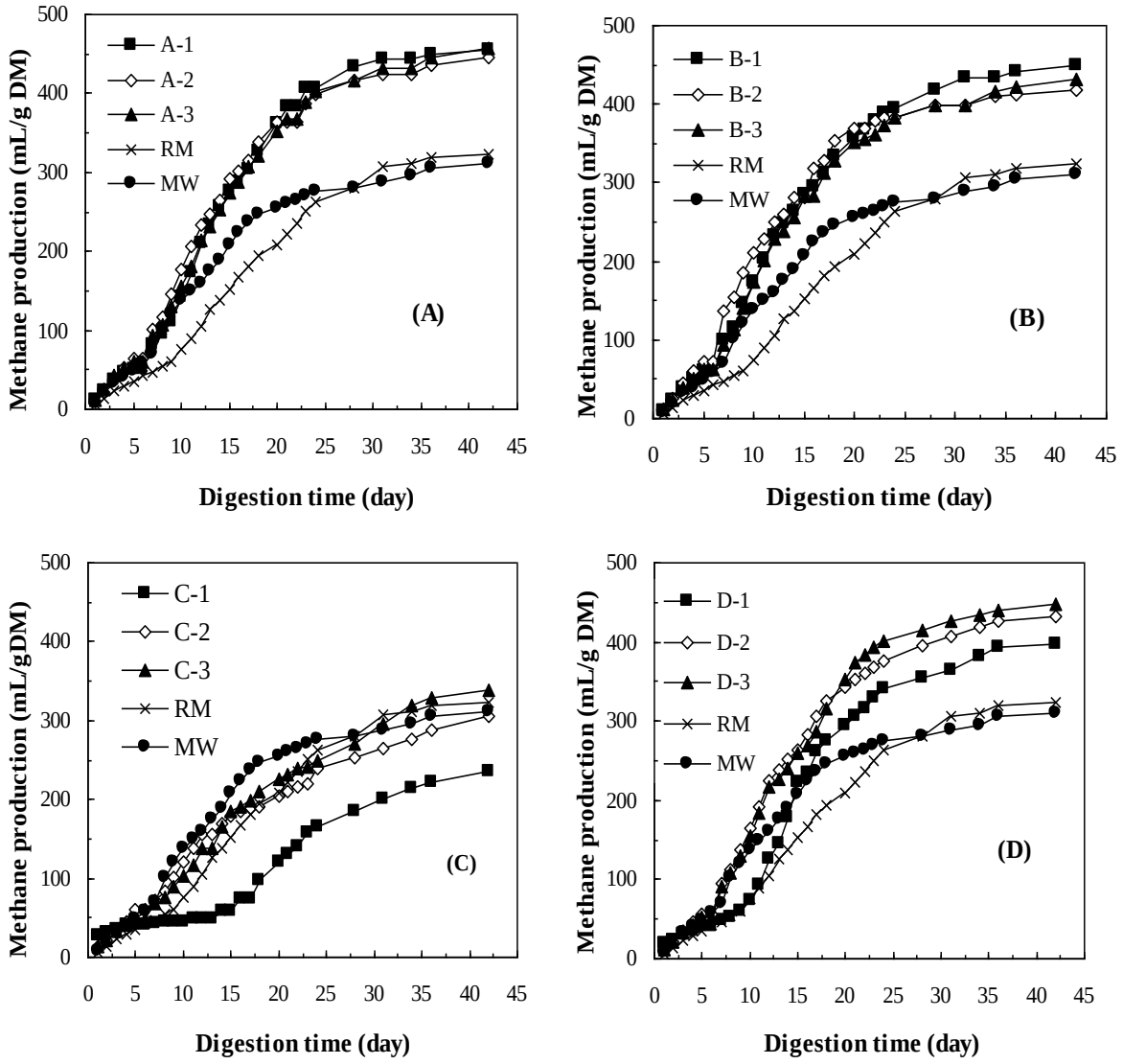


Fig. 3.3. Cumulative biogas production from manure treated by microwave-assisted pretreatment. (A-1) manure treated with high loading of NaOH, (A-2) manure treated with low loading of NaOH, (A-3) manure treated with low loading of NaOH and H_2O_2; (B-1) manure treated with high loading of CaO, (B-2) manure treated with low loading of CaO, (B-3) manure treated with low loading of CaO and H_2O_2; (C-1) manure treated with high loading of H_2SO_4,

(C-2) manure treated with low loading of H_2SO_4, (C-3) manure treated with low loading of H_2SO_4 and H_2O_2; (D-1) manure treated with high loading of HCl, (D-2) manure treated with low loading of HCl, (D-3) manure treated with low loading of HCl and H_2O_2. The biogas from raw manure (RW) and manure treated by microwave heating (MW) was also presented. Data are means of three replicates.

3.4.4. Struvite precipitation

Possible struvite crystal precipitated as a hard brown solid when magnesium was added to the liquid. The precipitated solid had a cylindrical-like crystalline structure (Fig 3.4). The precipitated solid from different treatments had a similar crystalline structure. Residual ortho-P concentrations were lower than initial ortho-P concentrations (Fig. 3.5), indicating struvite precipitation effectively removed ortho-P from the liquid. However, the ortho-P removal efficiency was dependent on the chemicals used in the pretreatment. The acid pretreatment (methods C and D) resulted in a higher ortho-P removal than alkaline pretreatment (methods A and B). Ortho-P removal from manure with the CaO-based pretreatment was particularly low (~40%). Manure treated by microwave heating without chemical addition (method MW) had ~50% ortho-P removal. For each chemical, adding H_2O_2 did not significantly improve in ortho-P removal compared with the H_2O_2-free treatment (Fig 3.5).

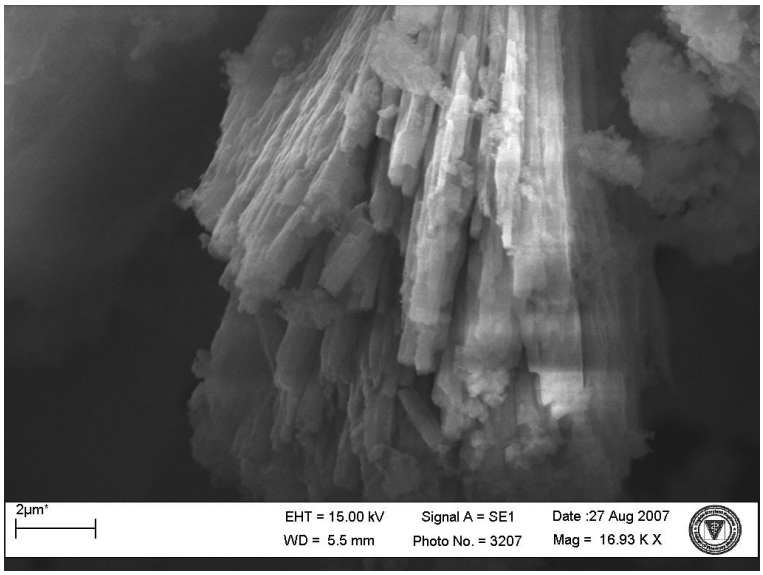


Fig. 3.4. SEM image of a possible struvite crystal.

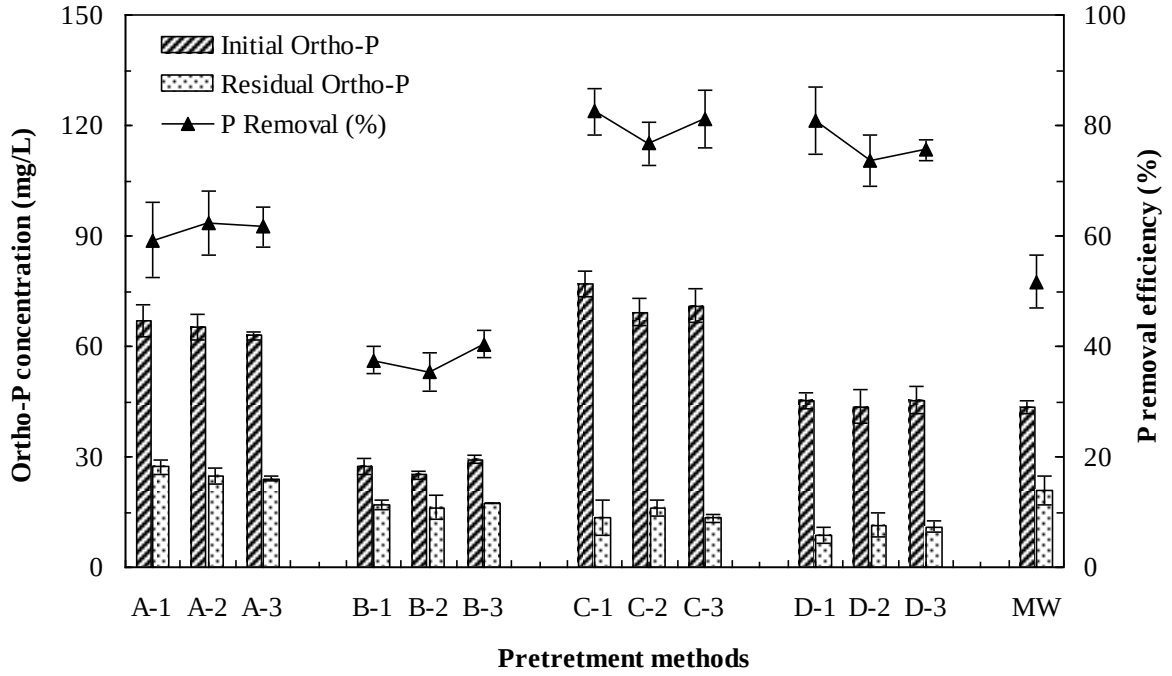


Fig. 3.5. Initial and residual ortho-P concentrations of the manure liquid solution before and after struvite precipitation, and the corresponding phosphate removal efficiency. (A-1) manure treated with high loading of NaOH, (A-2) manure treated with low loading of NaOH, (A-3) manure treated with low loading of NaOH and H_2O_2; (B-1) manure treated with high loading of CaO,

(B-2) manure treated with low loading of CaO, (B-3) manure treated with low loading of CaO and H_2O_2; (C-1) manure treated with high loading of H_2SO_4, (C-2) manure treated with low loading of H_2SO_4, (C-3) manure treated with low loading of H_2SO_4 and H_2O_2; (D-1) manure treated with high loading of HCl, (D-2) manure treated with low loading of HCl, (D-3) manure treated with low loading of HCl and H_2O_2. Data are means of three replicates and error bars show standard deviations.

3.4.5. Comparison of microwave-heating and conventional-heating

Microwave heating and conventional heating were used to treat dairy manure to evaluate the effects of the two heating methods on the pretreatment performance. Two chemicals were added in the pretreatment: NaOH (with 0.035 g/g DM loading) or HCl (with 0.185% loading).

For each chemical used, microwave heating resulted in more solubilization of lignocellulose than conventional heating, as indicated by less amount of glucan/xylan remaining in the microwave-pretreated manure (Table 3.5). In the liquid phase, concurrently, SCOD/TCOD ratio, soluble-P, ortho-P, and ammonium concentration from microwave-heated samples were higher than those from conventional-heated samples (Table 3.5). Struvite precipitation indicated the phosphate removal efficiency was also high in microwave-based pretreatment (data not shown). Manure heated by microwave produced more methane than the conventional-heated manure (Fig 3.6), suggesting a better anaerobic digestibility resulted from microwave heating. The two chemicals used in the pretreatment yielded similar biogas production (Fig. 3.6).

Table 3.5. Comparison of microwave- and conventional-heating pretreatment with two different chemical loadings [a]

Parameters	NaOH (0.035 g/g DM)		HCl (0.185 %, V/V)	
	Microwave	Conventional	Microwave	Conventional
Glucan+xylan (% of original)	76.2±1.1	91.0±2.1	93.6±1.3	97.0±0.9
SCOD/TCOD (%)	11.43±0.28	7.08±0.05	9.57±0.15	8.22±0.16
Soluble-P (mg P/g DM)	3.13±0.02	1.77±0.52	2.78±0.30	1.18±0.12
Soluble-P released (%)	35.04	11.28	28.91	0.91
Ortho-P (mg P/g DM)	1.84±0.10	1.33±0.14	1.27±0.13	0.87±0.09
Ortho-P released (%)	23.88	14.91	13.86	6.76
Ammonium (mg N/g DM)	8.10±1.72	6.20±0.69	8.64±2.29	6.55±0.78
Ammonium released (%)	9.29	2.83	11.09	3.99

[a] Data are means of three replicates ± standard deviations.

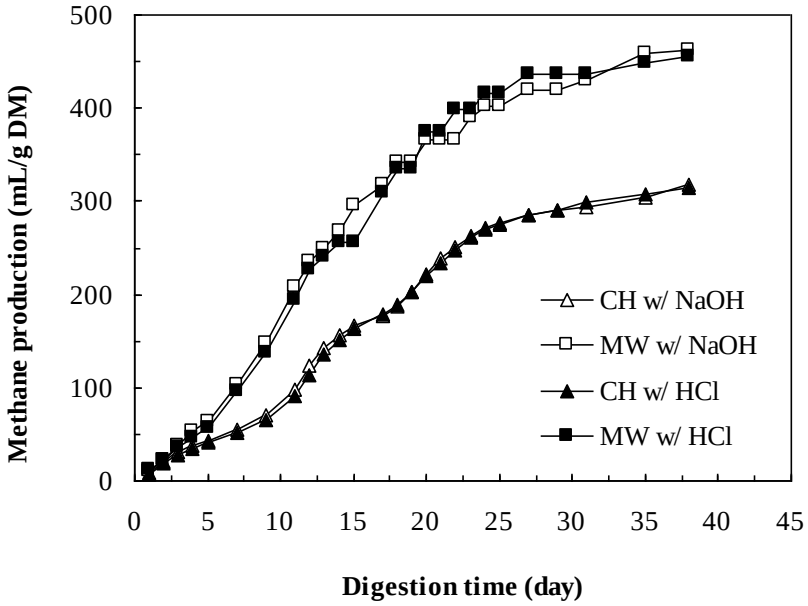


Fig. 3.6. Cumulative biogas production from manure treated by microwave-heating (MW) and conventional heating (CH) with different chemicals (NaOH with 0.35 g/g DM loading; HCl with 0.185% loading). Data are means of three replicates.

3.4.6 Optimization of temperature and heating time in microwave pretreatment

Temperature (T) and heating time (t) used in microwave pretreatment may influence the pretreatment performance. The two parameters were thus optimized as described in Section 3.2.2.2. The responses (ortho-P content and methane production) were correlated as functions of T and t, i.e.,

$$Ortho-P\ (mgP/gDM) = 1.95 + 0.22 \cdot T + 0.05 \cdot t + 0.25 \cdot T \cdot t - 0.31 \cdot T^2 - 0.16 \cdot t^2 \quad (3)$$

$$Methane\ \ (mL/gDM) = 441 + 16.28 \cdot T + 1.88 \cdot \cdot t + 4.50 \cdot T \cdot t - 10.36 \cdot T^2 - 5.61 \cdot t^2 \quad (4)$$

The correlation coefficient (R^2) for equation (3) and (4) was 0.87 and 0.85, respectively. Fig. 3.7 shows the surface responses of ortho-P content and methane production as functions of T and t, respectively. The plots are hump shaped with a clear peak, indicating the maximum solution can be achieved within the experimental range. The optimal values of T and t are listed in Table

3.7. The predicted value of ortho-P content and methane production at these conditions is also presented (Table 3.7). The ortho-P content had 10.1% deviation from the experimental value, while the predicted value of methane production had 1.8% deviation.

Table 3.6. Central composite design of temperature (T) and time (t) used in microwave pretreatment with methane production and ortho-P content as responses

	Variables				Responses	
	T		t		Methane	Ortho-P
Run	Coded unit	True value	Coded unit	True value	(mL/g DM)	(mg/g DM)
1	+1	150^{o}C	+1	30 min	445	2.22
2	+1	150^{o}C	-1	10 min	432	1.61
3	-1	90^{o}C	+1	30 min	402	1.10
4	-1	90^{o}C	-1	10 min	407	1.50
5	0	120^{o}C	+1.41	34 min	442	1.56
6	0	120^{o}C	-1.41	6 min	437	1.44
7	+1.41	162^{o}C	0	20 min	452	1.89
8	-1.41	78^{o}C	0	20 min	408	1.01
9	0	120^{o}C	0	20 min	452	1.94
10	0	120^{o}C	0	20 min	445	1.92
11	0	120^{o}C	0	20 min	435	2.00

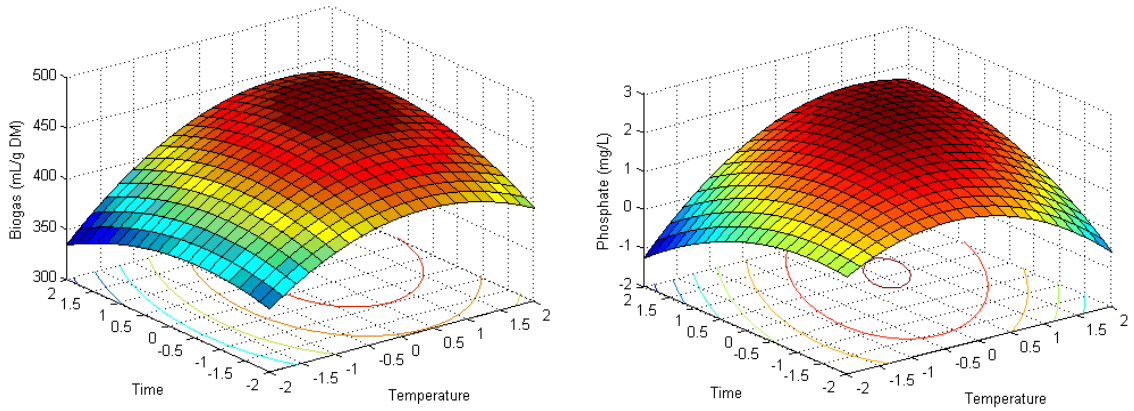


Fig. 3.7. The response surface of biogas (methane) production and ortho-P as functions of temperature and heating time used in microwave pretreatment

Table 3.7. Summary of optimal values of T and t used in microwave pretreatment for achieving maximum methane production and ortho-P release

	Optimal values		Predicted value	Experiment value	Error (%)
	T	t			
Methane	147 ^{o}C	25.3 min	451 mL/g DM	443 mL/g DM	1.81
Ortho-P	135.3 ^{o}C	26.0 min	2.03 mg P/g DM	2.26 mg P/g DM	10.17

3.5. Discussion

Pretreatment has been used to enhance the solubilization of various waste materials and thus, the performance of subsequent aerobic or anaerobic treatment of these materials. Most of these pretreatments used thermochemical methods (Azbar et al., 2008; Bougrier et al., 2006a; Bougrier et al., 2007; Eskicioglu et al., 2006; Lissens et al., 2004a; Mladenovska et al., 2006), although other methods such as mechanical disintegration (Nah *et al.*, 2000), ultrasound (Sangave *et al.*,

2007), electrochemical processing (Watanabe *et al.*, 2006), and ozone oxidation (Sievers *et al.*, 2004) have also been reported.

The results obtained in this study show that microwave-based pretreatment is beneficial to the solubility of the complex manure particles. Microwave based pretreatment has proved to be effective on enhancing solubility and anaerobic digestibility of sewage sludge (Eskicioglu et al., 2006; Park et al., 2004). The hypothesis is that microwave irradiation can disrupt the complex structure of activated sludge floc and release extracellular polymer substances, proteins, and sugars into a soluble phase (Eskicioglu *et al.*, 2006).

In addition to soluble COD that provided an indication of a general extent of organic carbon solubility (Fig. 3.1), the reduction of glucan/xylan of the treated manure (Fig. 3.2) indicate that microwave-based thermochemical pretreatment facilitated manure fiber degradation. The trends of SCOD/TCOD with different pretreatment methods correlated with that of glucan/xylan reduction. For example, the treatments C-1 and D-1 resulted in higher SCOD/TCOD ratios (Fig. 3.1), while the glucan/xylan reductions of these two treatments were also high (Fig. 3.2). Fiber as a major component in dairy manure is known for its recalcitrant nature and thus difficult utilization by native anaerobic organisms. In general, manure fiber is "tougher" than other lignocelluloses, as the easily hydrolyzed part is digested by the cattle. Microwave-assisted pretreatment is an effective way to break down the structure of lignocellulosic materials such as switchgrass (Hu and Wen, 2008) and rice straw (Zhu *et al.*, 2006). The reduction of glucan/xylan in the treated manure indicates that microwave assisted thermo-chemical pretreatment can also effectively break down manure fibers.

The COD solubilization and fiber degradation resulted in increased volatile fatty acids in the pretreated manure slurry (data not shown). These compounds may boost the methane production.

However, the trends of methane production with different pretreatment methods did not correlate with that of COD solubilization and fiber degradation. For example, the sulfuric acid-based treatment, particularly at high concentration, led to a much lower methane production (C-1, Fig. 3.3C) although SCOD/TCOD ratios and glucan/xylan reduction of acid-treated samples were high (Figs. 3.1 and 3.2). The reason for this phenomenon may be the presence of sulfur, which is toxic to anaerobic microbes particularly at high concentrations (Lens *et al.*, 1998). Another reason may be side reactions (such as Maillard reaction between amino acids and reducing sugars) during the acid treatment. Indeed, the BMP tests showed that the sulfuric acid-treated manure changed into dark black after a few days incubation, which is indication of the side reactions (data not shown).

The pretreatments also increased the solubilization of organic phosphorus and proteins, as indicated by the release of soluble-P, ortho-P, and ammonium from pretreated manures (Table 3.4). The soluble-P release ranged from 20-40% of total phosphorus, while ammonium release ranged from 10-14%. However, the CaO-based pretreatment (runs B) resulted in much lower ortho-P compared with other chemicals (Table 3.4). The reason was the presence of calcium, which can precipitate phosphorus through the formation of insoluble calcium phosphate (hydroxyapatite-HAP) (Ahn and Speece, 2006).

Microwave pretreatment has been used for phosphorus solubilization in municipal sewage sludge. Overall, ortho-P release from sewage sludge (Liao *et al.*, 2005) was higher than those observed from dairy manure in the current study. For example, sewage sludge heated by microwave at 120^{o}C for 17 min released 36.3% of ortho-P (Liao *et al.*, 2005), while manure treated by microwave heating reported here (Run MW Table 4) only released 12.91% ortho-P even at a longer heating time (30 min). This low release efficiency is likely due to the complex

structure of dairy manure. Sewage sludge is essentially microbial cells, which release phosphorus relatively easily when cells are disrupted. In contrast, dairy manure particles contained many forms of phosphorus including inorganic-P, acid soluble organic-P, lipid-P, and nucleic acid-type P (Barnett, 1994; Pan *et al.*, 2006), which are more difficult to solubilize. Adding H_2O_2 in the treatment did not significantly ($P < 0.1$) increase the phosphorus solubilization (Table 3.4), which is different from the previous report (Pan *et al.*, 2006). This compound was expected to increase phosphorus solubilizaion because tydroxyl free radical was produced in the treatment and may enhance the effectiveness of the process. The reason may be due to the low dosage of H_2O_2 used in this study.

Once ortho-P was released, struvite precipitation proved an efficient way to recover the phosphate from the liquid phase (Fig. 3.5). The crystalline structure (Fig. 3.4) confirms that the precipitated solid was struvite crystal because other types of phosphorus (e.g., hydroxyapatite) are commonly amorphous (Ahn and Speece, 2006). It should be noted that optimizing struvite precipitation conditions can further enhance phosphate removal efficiency. For example, struvite precipitation was more efficient at pH 9 than that at pH 8 (data not shown) because high pH usually results in more ortho-P existing in the solution than monohydrogen phosphate and dihydrogen phosphate.

CaO-based pretreatment (B-1, B-2 and B-3) had low struvite precipitation efficiency (Fig. 3.5). This was likely due to low initial phosphate available for struvite formation as phosphate was precipitated by calcium during the pretreatment stage.

The comparison of microwave- and conventional-heating treatment demonstrates that microwave pretreatment was more effective in facilitating manure solubilization (Table 3.5) and digestibility (Fig. 3.6). The benefit of microwave heating was due to the unique feature of

microwave irradiation (de la Hoz *et al.*, 2005). When heterogeneous manure slurry was microwave irradiated, the polar substances in the slurry were heated more rapidly than non-polar substances. This causes temperature inhomogeneity at the microscopic level. This unique feature eventually results in an "explosion" effect among the particles, and improves the disruption of the physical structures of manure particles. In contrast, conventional heating was slow and transferred the heat into the sample from surface, resulting in less structural change (Tokuyama and Nakamura, 2005).

The industrial use of microwave heating is mostly in food process such as drying and cooking. The microwave heating can save 40-50% of electricity by faster heating, and the maintenance cost of microwave heating is lower because the heating process is cleaner and can be instantaneous controlled. However, the initial cost of setting up the microwave generator and heating chamber is very high. In our study, microwave heating can produce 30% more biogas and release 38% more ortho-phosphorus, combined with the lower operation and maintenance cost, microwave heating is a potential energy save technology.

In the optimization of temperature and heating time used in the microwave-based pretreatment, the optimal values of the two parameters were within the experimental range. The surface response results show that both ortho-P content and methane production did not fall off steeply if temperature and heating deviated from their optimal values (Fig. 3.7). These are desirable properties because it means that even if the operational parameters fluctuate, the performance of microwave pretreatment will be robust despite fluctuations in the operation parameters, without a significant decrease.

3.5. Conclusions

Microwave-based thermochemical pretreatment was an efficient way to increase anaerobic digestibility and phosphorus recovery through enhanced solubilization of manure components. The following conclusions can be drawn: first, Microwave-based thermochemical (sodium hydroxide, calcium oxide, sulfuric acid or hydrochloric acid) pretreatment enhanced COD solubilization, glucan/xylan degradation, phosphorus and ammonium solubilization; second, When sulfuric acid is used in the pretreatment, anaerobic digestibility of pretreated dairy manure was reduced, probably due to inhibitory effects of sulfur and Maillard side reaction; third, Calcium oxide-based pretreatment led to a lower ortho-P release, and thus, low struvite precipitation efficiency, which was caused by the formation of calcium phosphate; fourth, Sodium hydroxide- and hydrochloric acid-based pretreatment simultaneously enhanced anaerobic digestibility and struvite precipitation efficiency. Using these two chemicals, the microwave heating resulted in a better manure solubility and anaerobic digestibility than conventional heating; last, within the experimental range studied, the phosphorus solubilization and anaerobic digestibility of microwave-pretreated manure is robust to fluctuations of the temperature and heating time.

In order to fully realize the potential benefits of microwave pretreatment, future works should focus on system scale-up using batch or continuous processes. Moreover, a critical assessment of cost analysis is needed.

Chapter 4 DNA Based Molecular Assessment of Pathogenic Organisms in Dairy Manure

4.1 Introduction

Pathogen contamination of water resources is a risk to human health. This contamination originates from various sources such as wildlife, urban runoff, landfills, sewer overflow, and animal waste (Pathways and Monitoring in Natural and Engineered Systems, 2007). Understanding the sources, fate, and transport of pathogens in water and wastewater treatment systems and various watersheds is important to protect food safety, keep a sustainable natural resource and environment, and improve the quality of human life.

Escherichia coli are part of the normal flora of the human and animal gut, and can benefit their hosts by producing vitamin K2 (Bentley and Meganathan, 1982), or by preventing the establishment of pathogenic bacteria within the intestine (Reid *et al.*, 2001). However, certain strains of *E.coli* can cause disease in humans and animals. *E.coli* O157:H7 can cause bloody diarrhea and 10-20% of patients can develop more severe diseases such as hemorrhagic colitis, hemolytic uremic syndrome, or thrombocytopenic purpura (CDC, 1997). Most of the outbreaks are linked to the consumption of contaminated and undercooked bovine food products (Feng, 1995). However, some of the outbreaks are traced to animal manure, and a Canadian study showed that there is a significant spatial association between beef cattle density and human shiga toxin-producing *E.coli* infection (Kudva *et al.*, 1998).

Development of a robust method to accurately assess pathogens in manure matrices is needed. Currently, most of the techniques employed for pathogen detection are culture-based measurement. However, several major drawbacks are connected with these culture-based techniques. Most importantly, the determinations is time-consuming which require one to several

days to obtain results, for some zoonotic pathogen(pathogens can be transmitted from animal to human) like *M. paratuberculosis,* it takes even longer (8-16 weeks) and cultures are often lost due to the contamination of other microorganism(Grewal *et al.*, 2006). This could be critical if decisions on eventual measures require quickness and preciseness. In addition, cultivation techniques frequently lack sufficient specificity, false-positive results are found in many cases and subsequent assay such as immunoassay are required (Aitken *et al.*, 2005). On the other hand,

pathogen that undergoes severe environmental stress such as nutrient deprivation, metals, chlorine, low temperature could enter a state called "viable but non-culturable" (VBNC), the stage was described as one where bacterial cells are intact and alive when tested for criteria such as enzyme activity, photosynthesis, respiration, and energy charge, but do not undergo cell division on routinely employed bacteriological media (Higgins *et al.*, 2007). However, VBNC pathogen can be recovered and become virulent in their natural host (Dinu *et al.*, 2009). A more comprehensive approach which could quickly and precisely track the pathogen is urgently needed.

Molecular-based methods such as quantitative PCR (qPCR) have been employed for pathogen measurement. DNA-based quantitative PCR has been well-developed for municipal biosolid by Chen et al. in 2007, and the method is proved to be able to track and enumerate *E.coli* that are undetectable by culture-base measurement(Chen *et al.*, 2006; Higgins *et al.*, 2007). However, dairy waste is an extremely complex matrix of which consists large amount humic acid and protein, and those compounds could have significantly negative influence on PCR. Moreover, the extremely complex matrix can also block the reagents from cell lysis and entrap the DNA from extraction and the efficiency would be influenced as well. Chen et al mixed a known DNA template with the solid and extract them together. However, the lysis

efficiency is still underestimated. To overcome these limitations, a control strain was constructed and used as an internal control for the detection of efficiency. The objectives of this work are to develop a molecular tool based method for pathogen identification and quantification, and then compare the new developed method with the traditional culture-based method.

4.2 Hypothesis

Our hypothesis is: first, *E.coli* tends to grow on solid surface in environment, and thus the subsequent formation of biofilm will shelter cells from culture based detection, causing underestimation of *E.coli* number. Sonication can help recover attached bacteria from sediments. Second, the molecular tool based method has advantage over culture-based method.

4.3 Methods and materials

4.3.1 Sample collection and analysis

The sample collection was conducted as described in section 3.3.1.

Raw manure was first diluted with tap water to reduce the solid content to 1% (w/w) with a total volume of slurry being 500ml. The slurries were then sealed in a 1L glass bottle; nitrogen gas was used to purge the reactor to remove oxygen. The glass bottle was then incubated in shaker at 37 ^{o}C. The hydraulic retention time of the digester was 22 days with 22.7 ml slurry was withdrawn from the digester, while the same volume of fresh manure slurry was fed to the digester on a daily basis. .

Total solid (TS) and volatile solid (VS), were determined according to standard methods (APHA, 1995), bio- methane was estimated by mixing the biogas with 1N NaOH solution and

recording the gas volume after CO_2 absorption. *E.coli* numbers in fresh manure, MAD effluent were determined by the culture-based and molecular based methods described below.

4.3.2 Enumeration of *E. coli* using culture-based methods

The *E.coli* number in the samples was determined by U.S. Environmental Protection Agency (USEPA) Approved Method #1603. Although the method has been successfully applied to manure samples by Weaver et al (Weaver *et al.*, 2005), its accuracy and precision remains controversial, whether this standard method could detect both attach and unattach E.coli in high solid contene samples is unknown. To prove our hypothesis as described in section 4.2, ultra-sonication was employed for shaking cells off from solid surface. Different treatments regarding the sonication time were tested, and the final treatment was described as below. The materials and protocol from Difco Laboratories (Detroit, MI) were used to implement the EPA 1603 methods. In brief, 1 g manure sample was resuspended in phosphate buffer solution (NaH_2PO_4 0.58g/L, Na_2HPO_4 2.5g/L and NaCl 8.5g/L, pH 7.2) and stirred. The mixture was then diluted in serially with phosphate buffer solution. The final diluted solution was sonicated (VWR symphony Ultrasonic cleaner) for 5 minutes to improve distribution of *E.coli*, and then filtered through a 0.45 μm membrane (mixed cellulose esters, Millipore). After filtration, the membrane was placed on modified membrane-thermo tolerant *Escherichia coli* agar plates (modified mTEC). The plates were first incubated at 35 ^{o}C for 2 hours, and then at 44.5 ^{o}C for 22-26 hours. After incubation, the red or magenta colonies that appeared were counted.

4.3.3 Gene disruption of *E. coli* BW25113

gadA and *gadB* has been identified as a suitable target in quantitative PCR experiments for

estimating *E. coli* population density in environmental samples (Grant *et al.*, 2001). In our studies, the *gad*A and *gad*B genes were used as PCR targets in the *E.coli* detection. The control strain in the molecular based method serves as an internal control. To accomplish the goal, the *E.coli* quantification target gene *gad*A and *gad*B should be removed from chromosome DNA of the control strain. Meanwhile, the inserted kanamycin resistance gene (*kan*) could be used to quantify the number of added control strain cells. Therefore, the strategy of strain construction is to eliminate both *gadA* and *gadB*, and then permanently insert kanamycin resistance cassette.

The strategy of gene disruption is similar as shown in Figure 4.1. However, since the final goal was to permanently insert the kanamycin resistance cassette, the presence of FRT cassette might potentially decrease the stability of inserted kanamycin resistance gene. Therefore, the disruption steps were conducted by PCR product amplified without two FRT cassettes.

100 ng of purified PCR product without FRT cassettes was gel purified and suspended in elution buffer (10 mM Tris, pH 8.0) and then electrotransformed into the BW25113 Δ*gadA* (Fig 4.1 step 1). To increase the specificity of insertion and make the PCR product more precisely recombine to target, The P1and P2 primers size were designed as 40 bases long instead of 20 bases long.

The BW25113 Δ*gadA* with a Red helper plasmid were grown in 5 ml SOB cultures with ampicillin and L-arabinose at 30 ℃ to an OD600 ~0.6. In the original paper, L-arabinose was added when competent cells grow to an OD600=0.6, followed with 6 hours induction. The purpose of adding L-arabinose latter is that cell may have unexpected mutant with long time exposure of L-arabinose. However, in our case, the original method cannot provide enough enzymes induced by red plasmid for the gene interruption. Therefore, L-arabinose was added simultaneously with competent cell cultivation to achieve better amount of enzyme.

Competent cells were electroporated with purified PCR product. Shocked cells were added to 1 ml SOC incubated 1 h at 37 ℃, and then one-half was spread onto LB plate with kanamycin to select kanamycin insertion. If none grew within 24 h, the remainder was spread after standing overnight at room temperature. After primary selection, mutants were patched onto LB plate without kanamycin; the colonies were incubated at 37 ℃ overnight, and then tested for ampicillin sensitivity for loss of the red helper plasmid. If it was not lost, then a few were colony-purified once at 43 ℃ and similarly tested. (Datsenko and Wanner, 2000).

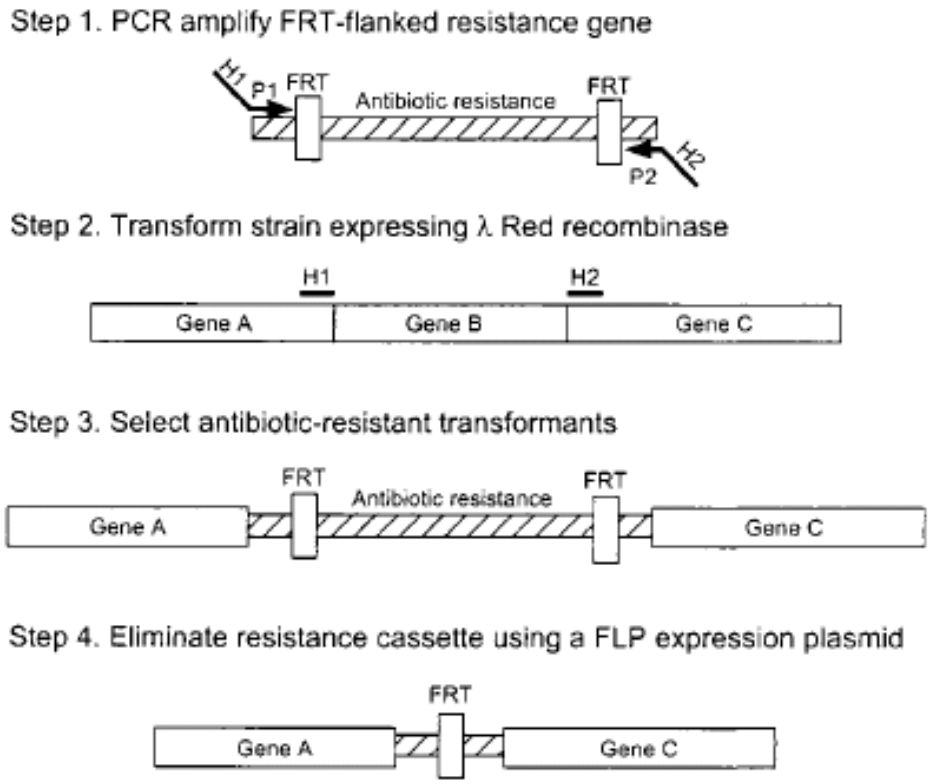


Figure 4.1 Gene disruption strategies (Datsenko and Wanner, 2000). H1 and H2 refer to the homology extensions or regions. P1 and P2 refer to priming sites.

4.3.4 PCR verification

To verify whether the kanamycin resistance cassette was successfully transformed into BW25113 Δ*gadA*, selected colonies were subjected to PCR verification process. In order to prove that the target cassette was inserted into the correct site, the forward primer was designed at the upstream of *gadB* gene. To ensure that the kanamycin resistance cassette was inserted, the reverse primer was designed in the middle of the cassette (Fig 4.2). A portion of patched colonies were suspended in 20 µl sterile water, and 5 µl portions were used in PCR amplification.

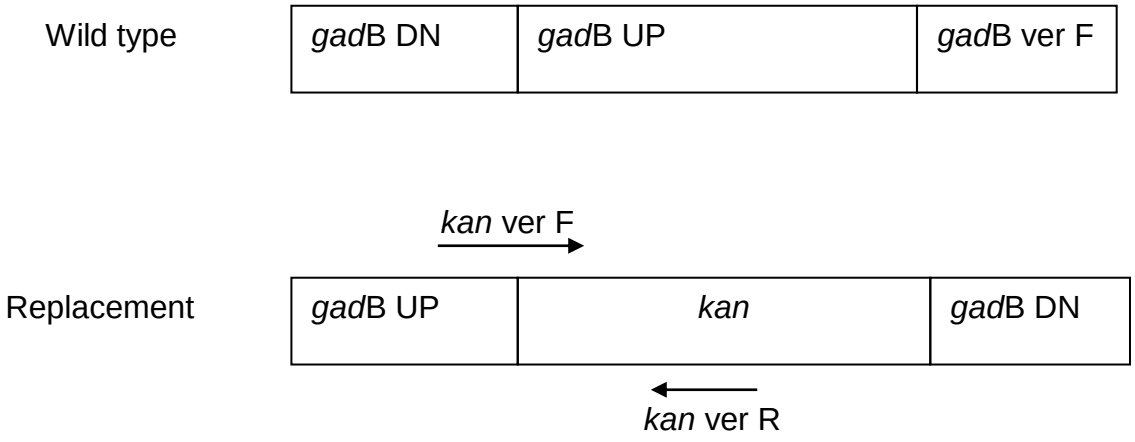


Figure 4.2 Illustration of *gad*B disruption product verification procedure.

In order to verify that the final control strain has the correct cassette at the right gene sites, further PCR verification and sequencing were performed. For both *gad*A and *gad*B, the forward primer was located at the upstream of each gene, and the reverse primer was located at the downstream of each gene (Fig 4.3).

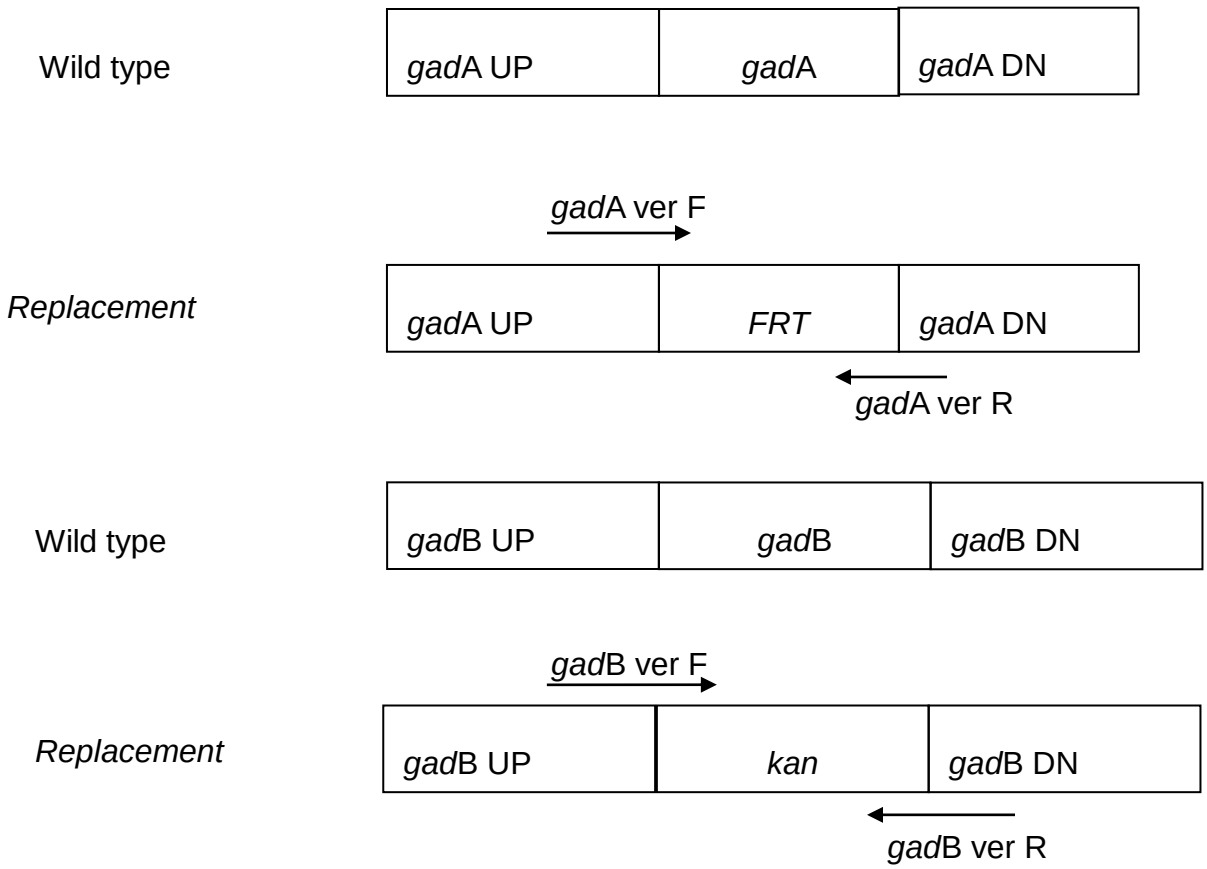


Figure 4.3 Illustration of *gad*A/*gad*B verification primer design

4.3.5 DNA extraction

The solvent-based extraction protocol was based on Fuentes' paper (Arbeli and Fuentes, 2007) with some modifications (Eric F. Johnson and Biswarup Mukhopadhyay, unpublished information). In brief, 0.5 g of manure or slurry was weighed and 3 mL of lysis buffer (100mM Tris–HCl, 100mM Sodium EDTA, 100mM Na_2HPO_4, 1.5M NaCl, and 1% hexadecylmethylammonium bromide (CTAB, pH= 8)) was added to the samples. Samples were vortexed with 1 g of 1 mm glass beads and 50 µl of lysozyme (100 mg/ml) for 15 sec. The mixture was incubated at 37 ^{o}C for 30 min, then protease K and DTT was added and the mixture was incubated at 37 ^{o}C for another 30 min. The mixture was mixed with 400 µl 20% SDS and

incubated at 65 ^{o}C for 1 hr. After incubation, the samples were centrifuged at 6000 ×g for 5 min and the supernatant was placed in a clean tube. The remaining pellets were re-extracted by lysis buffer and 20% SDS for 10 min, and the mixture was centrifuged at 6000 ×g for 5 min. Supernatants of the two extractions were combined and subjected to further PCI (Phenol/choroloform/isoamyl alcohol) and CI (choroloform/isoamyl alcohol) purification.

4.3.6 Enumeration of *E. coli* by real-time PCR

gadA and *gadB* has been identified as a suitable target in quantitative PCR experiments for estimating *E. coli* population density in environmental samples (Grant *et al.*, 2001). In our studies, the *gad*A and *gad*B genes were used as PCR targets in the *E.coli* detection. As metioned in section 4.3.3, the designed strain *E. coli* BW25113 Δ*gadA* ⊿ *gadB::kan* lacked those target genes allowing it to be distinguished from the wild type *E.coli* present in dairy manure and anaerobic digester effluent. The inserted *kan* gene allowed the quantification of added control strain numbers to determine the detection efficiency (an co efficiency represents the loss of DNA during extraction and inhibition factor in PCR reaction).

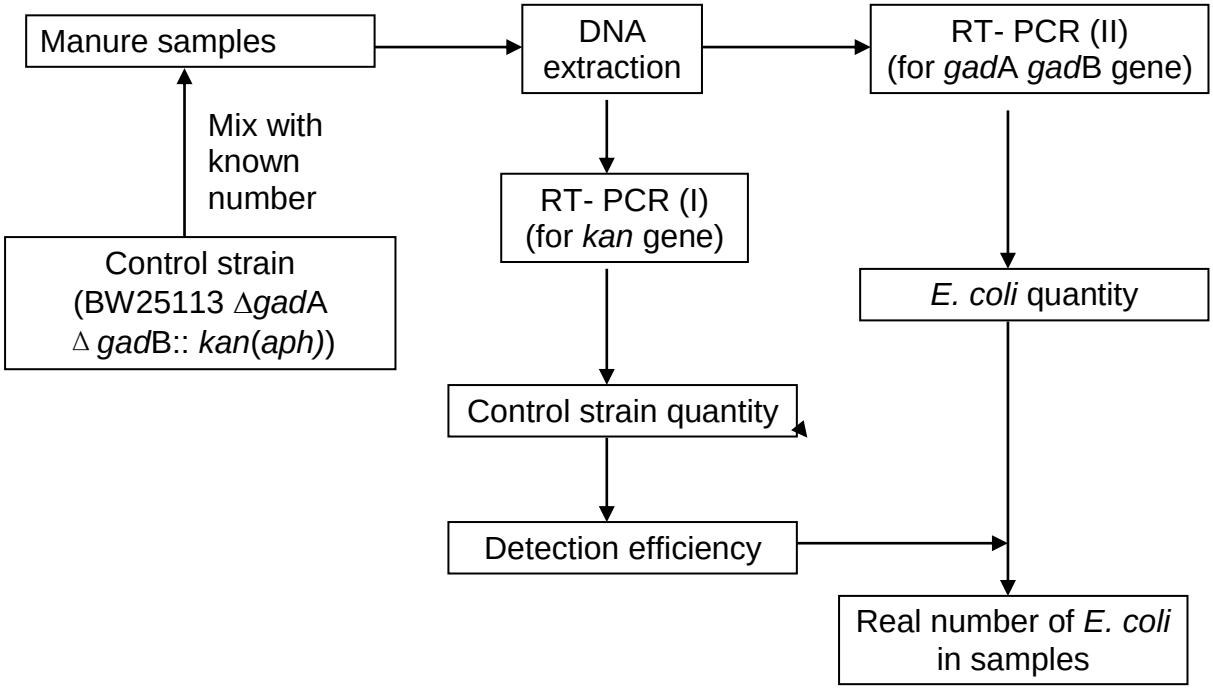


Fig 4.4. *E.coli* quantification method in dairy manure samples

Manure or anaerobic digesters effluent samples were mixed with pure control strain *E. coli* BW25113 Δ*gadA* ⊿ *gadB::kan* (Fig 4.4*)*. Subsequent DNA extraction was carried out by the method described in section 4.2.4. A multiplex real-time PCR was then performed to determine the Ct values (the number of cycles) of each gene, where the control (*E. coli* BW25113 Δ*gadA* ⊿ *gadB::kan*) and the target organisms in the manure (*E. coli*) were detected and quantified simultaneously. From the Ct values for *kan* gene, the number of control cells added to the sample was calculated from a standard curve developed by pure culture of the strain. By comparing the calculated number with actual number of cells, detection efficiency (a co efficiency of both DNA extraction and PCR effectiveness) would be obtained. After the *E. coli* number was calculated from *gadA/gadB* Ct value with a standard curve for the pure culture of BW25113, the number

was further corrected by the detection efficiency to determine the final value of real *E. coli* cells in samples.

4.3.7 Multiplex real- time PCR assay

The quantitative PCR performed by Taqman® system (Applied Biosystems, Inc) allowed the simultaneous measurements of *gadA/gadB* and *kan* genes. In Taqman® system, each gene requires a pair of primers and one probe of which consists an oligonucleotide with a 5' reporter dye and a 3' quencher dye were designed. During the PCR reaction, the cleavage of the probe by DNA polymerase separates the reporter dye and the quencher dye, which results in an incensement of reporter fluorescence. Ct value is determined by monitoring the fluorescence of the report dye.

The primer and probe sequences used to amplify *gadA/gadB* and *kan* are listed in Table 4.1. Probes were labeled with the reporter dye 6-carboxyfluorescein (FAM) or VIC at the 5' end. The quenchers were tagged with a minor groove binder (MGB) at the 3' end. Primers and probes were synthesized commercially by Applied Biosystems (Foster City, CA, USA). One thing to be noted is that *gad*A and *gad*B genes, which are identical at the coding sequence level, were detected by the same primer pair (Table 4.1) due to the high similarity. Therefore, they were quantified as a single target.

Table 4.1. Multiplex real- time PCR *gadA/gadB* and *kan* primers and probes

Primer or probe	Sequence (5'-3')	Tm (℃)	Location within the target gene	Size (bp)	Gene detected
*gad*A/*gad*B forward	GTATCGACATCGACATGCACATC	58.3	704-727	127	*gad*A &
*gad*A/*gad*B reverse	AATTTATGGCCTGAAGCACTGATC	58.2	806-830		g*ad*B
*gad*A/*gad*B probe	TGGCTTCCTGGCACCGTTCGT	65.2	743-764		
kan forward	GGGACTGGCTGCTATTGGG	58.3	274-293	147	*kan*
kan reverse	TCGCTTGGTGGTCGAATGG	57.9	401-420		
kan probe	TCCTGTCATCTCACCTTGCTCCTGC	65.4	318-343		

DNA was mixed with PCR master mix in 96-well micro optical plates (Applied Biosystems). All multiplex qPCR reactions were performed with 200 ng of template, 25 μl of multiplex PCR master mix (Applied Biosystems), primers (600 nm for *gadA/gadB* and 200 nm for *kan*) and probes (50 nm) in a final volume of 50 μl. Thermal cycling and data analysis for assays were performed with an ABI Prism 7300 Real-time PCR System (Applied Biosystems) using ABI sequence detection software. Water was used as a no-template control. All qPCR assays were carried out in duplicate, and qPCR efficiencies for each target were determined from the slopes of standard curves generated by plotting graphs of genomic DNA concentrations against Ct values.

4.3.8 Statistical analysis

The mean values and standard variation of the experimental results was calculated by Microsoft excel 2003. Statistical significance of the experimental data was tested using one way analysis of variance (ANOVA) by JMP. Tukey's pairwise comparisons were used to test differences between treatments.

4.4. Results

4.4.1 Optimization of culturing methods

No *E.coli* was found in TAD effluent, and TPAD effluents only had about 2-3 log number of *E.coli* (Figure 4.5). Compared to the qPCR results, the values were very low.

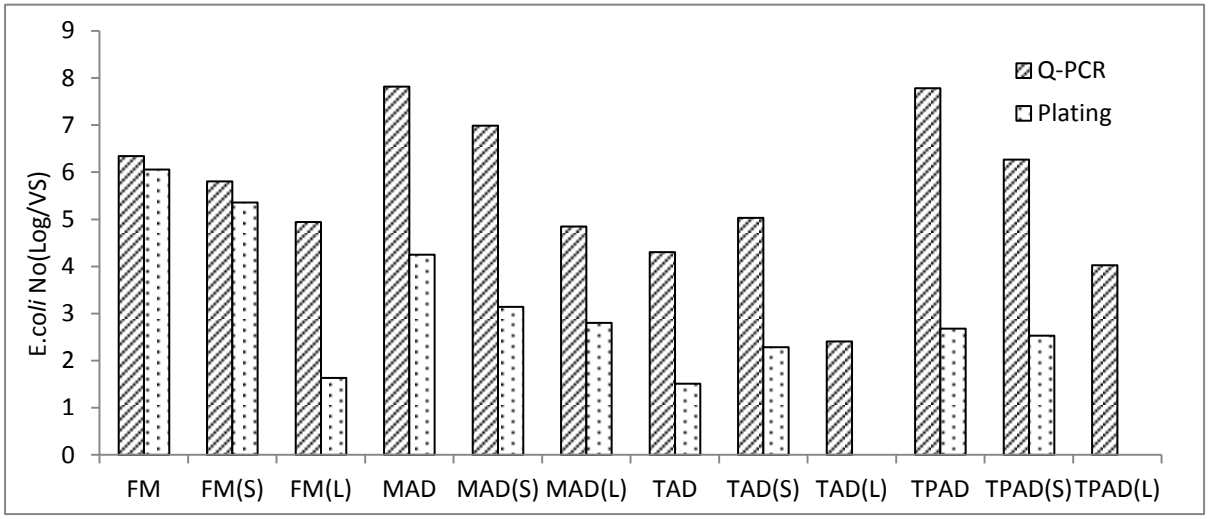


Figure 4.5 Fresh manure and anaerobic digestion samples *E.coli* numbers by unmodified plating method and real-time PCR

Table 4.2 Sonication test on pure culture cells BW25113 and BW25113 *ΔgadA ΔgadB::kan*

	Treatment 1[a]	Treatment 2[b]	Treatment 3[c]	Treatment 4[d]
BW25113(CFU/ml)	$5.22\times10^{9}\pm 9.22\times10^{8}$	$1.83\times10^{9}\pm 3.53\times10^{8}$	$1.66\times10^{9}\pm 7.64\times10^{7}$	$5.56\times10^{9}\pm 1.09\times10^{9}$
BW25113*ΔgadAΔgadB ::kan*(CFU/ml)	$7.92\times10^{9}\pm 1.22\times10^{9}$	$1.2\times10^{9}\pm 5.77\times10^{7}$	$1.58\times10^{9}\pm 2.47\times10^{8}$	$8.52\times10^{9}\pm 1.26\times10^{9}$

[a]. Control group: cells were only stirred during serial dilution

[b]. Cells were sonicated for 2.5 minutes at each step of dilution

[c]. Cells were sonicated for 5 minutes at each step of dilution

[d]. Cells were sonicated for 5 minutes only at the last step of dilution

E.coli strain and control strain in treatment 2 (2.5 minutes sonication at each dilution) and treatment 3(5 minutes sonication at each dilution) reduced cell number to about 1/3 and 1/6, respectively (Table 4.2). In contrast, in treatment 4 (one time 5 minutes sonication) cell numbers increased about 6-7%. These results suggest that long time serial sonication causes severe damage to cells; but one time 5 minutes operation would help even distribution of cells.

Table 4.3 Sonication test on fresh manure samples mixed with BW25113 *ΔgadA ΔgadB::kan*

	Treatment 1[a]	Treatment 2[b]	Treatment 3[c]
E.coli (CFU/g FM)	$9.2\times10^5 \pm 2.6\times10^5$	$12.6\times10^5 \pm 3.91\times10^5$	$11.9\times10^5 \pm 2.11\times10^5$
Control strain (CFU/g FM)[d]	$6.9\times10^5 \pm 1.54\times10^{5A}$	$9.1\times10^5 \pm 2.12\times10^{4B}$	$3.1\times10^5 \pm 1.72\times10^{5C}$

[a] Control group: cells were only stirred during serial dilution

[b] Cells were sonicated for 5 minutes only at the last step of dilution

[c] Cells were sonicated for 5 minutes at each step of dilution

[d] Tukey's pairwise comparisons was performed and values with different letters are significantly different ($p< 0.05$)

Compared with control (treatment 1), both treatment 2 (one time 5 minutes sonication) and treatment 3 (5 minutes sonication at each dilution) had significantly larger number of wild type *E.coli* than treatment 1(Table 4.3). In the control strain, cell numbers in treatment 3 was reduced by 50% whereas cell numbers in treatment 2 increased by about 50%. These data indicates that proper sonication is necessary for culturing method; longer sonication is detrimental to both control strain and wild type *E.coli*.

4.4.2 Strain construction

The construction of the control strain started from BW25113 (Δ *gad*A::Kan) which was bought from the Coli Genetic Stock Center at Yale. An ampicillin and Cm^R plasmid pCP20 was transformed into the start strain BW25113 (Δ *gad*A::Kan) (Figure 4.4). The thermal induction of Flp recombinase at 43 ^oC resulted in loss of FRT-flanked kanamycin resistance cassette and the FLP helper plasmid pCP 20 simultaneously. The genotype of the strain changed from BW25113 (Δ *gad*A::Kan) to BW25113 (Δ *gad*A::Kan) (Figure 4.6 step 2).

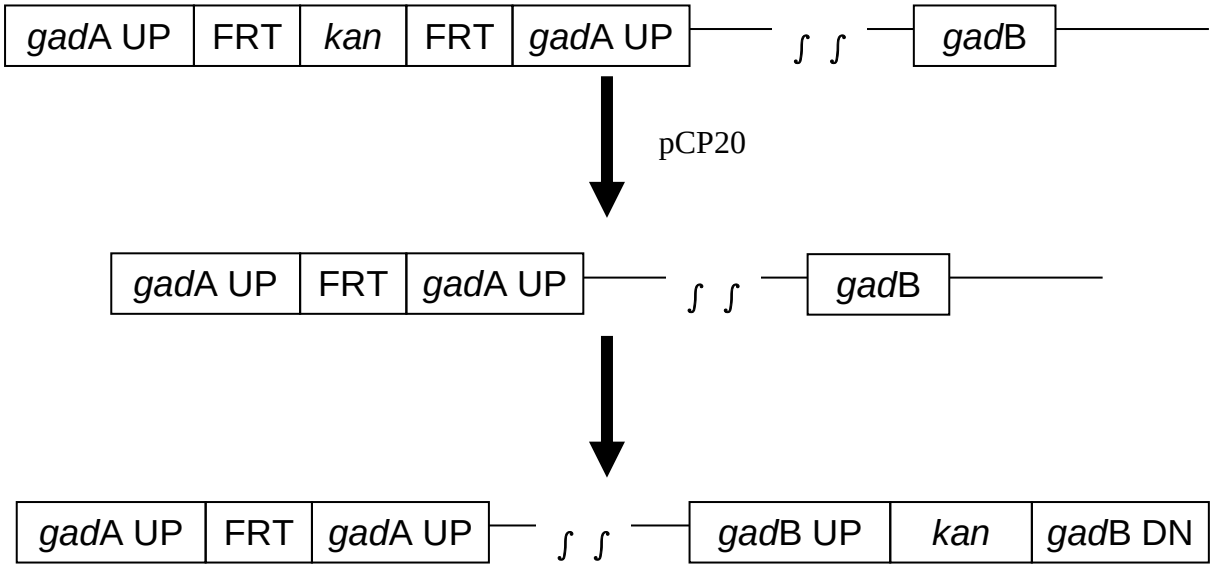


Fig 4.6. Construction of control strain BW25113 Δ*gadA ⊿ gadB::kan*

Table 4.4 Primer sequences of *gadB* gene disruption PCR product

Primer	Sequences
*gad*B/H1P1	5’ – TTA AGG AGT TCG AAA TGG ACG TGT AGG CTG GAG CTG CTT CG -3’.
*gad*B/H2P2	5’ – CAA CGT TAT GTT ATC AGG TGC ATA TGA ATA TCC TCC TAA G -3’

The 20 bases long P1and P2 primers (Table 4.4) were firstly transformed and the selected colonies were tested by PCR verification. The correct size of PCR product should be about 120 base pairs. However, no PCR product showed at 120 base pairs position (Fig 4.7a). Two bands at the bottom are diprimers indicating that gene disruption failed.

In the subsequent batch, H1P1 and H2P2 primers were designed as 40 bases long and same PCR verification was performed to test the selected colonies. All selected colonies generate correct size of PCR product (Fig4.7b), indicating the gene interruption was successful.

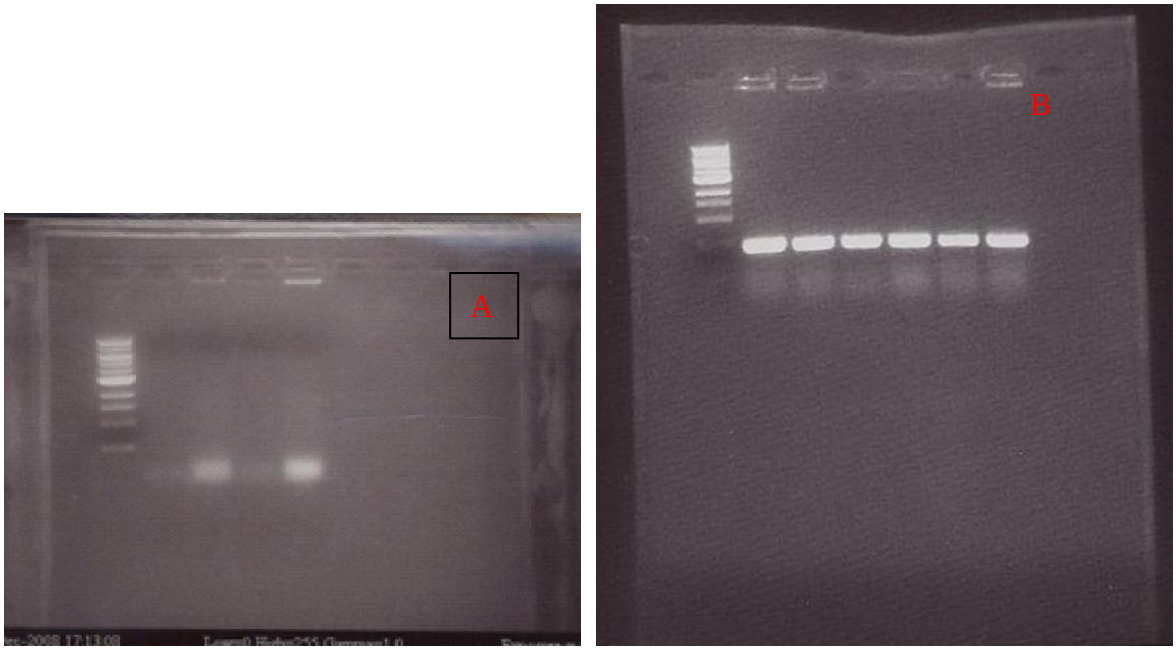


Figure 4.7 Gel images of kanamycin resistance cassette verification PCR product

Finally, in order to verify that the correct cassette was inserted at the right gene sites. Further PCR verification and sequencing were performed. By comparing the full sequences generated by PCR amplification to FRT and kanamycin cassette, both inserted genes show high similarity with desired genes (Table 4.5), indicating the control strain BW25113 Δ*gadA* ⊿*gadB::kan* was successfully constructed.

Table 4.5 Sequencing results of PCR verification product

Gene	Comparison Sequence		
*gad*A	Control strain	ATCCGTCGACCTGCAGTTCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCGAAGCAG	60
	FRT	----------GAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAG	41
		********** ** * * ** ********** * **	
	Control strain	CTCCAGCCTACAAACAGCTTTAAACACACCTGATAACATAACGTTGTAAAAACCGAATGC	120
	FRT	GAACTTC----------	48
	control strain	----------GAGGTTCTCAGGCGG-TTAGGTG-AGAGGTAAGTCG-CTATGCT--G	42
	kan	GATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGG	480
		******** *** * ** **** ***** * *** ***** *	
	control strain	GCACAGCAGACA-TTGGCTGTT-TGAAGCCGCCGTGTTCCGG-TGTCAGCGCAGGGGCGC	99
	kan	GCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGC	540
*gad*B		----------	
	control strain	ACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCA	938
	kan	ACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCA	1377
		**********	
	control strain	GCT-CAAAAGCGCTCTGCTATACTAGCA----------	965
	kan	GCTTCAAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAA	1437

4.4.3 Multiplex real- time PCR optimization

Three major factors would influent the Ct value of certain gene, especially in multiplex assay: the ratio of forward and reverse primer, the primer concentrations and probe concentration. In our studies, each of the factors was optimized by pure culture cell DNA.

The ratios of each primer were first tested. Ct values did not markedly vary under different ratios (Table 4.6), therefore, for easier preparation, same amount of forward and reverse primer were applied in the following tests.

Table 4.6. Multiplex real- time PCR forward and reverse primer ratio optimization [a,b]

Sample	Ct (*gadA/gadB*)	Ct (*kan*)
Forward/Reverse 200/200nM	21.07 ± 0.31^{A}	17.92 ± 0.04^{A}
Forward/Reverse 200/400 nM	19.87 ± 0.16^{B}	17.82 ± 0.04^{A}
Forward/Reverse 400/200 nM	21.74 ± 0.09^{C}	17.90 ± 0.10^{A}
Forward/Reverse 400/400 nM	20.14 ± 0.19^{C}	17.56 ± 0.09^{B}

[a] Data are means of three replicates ± standard deviations

[b] Tukey's pairwise comparisons was performed and values with different letters are significantly different ($p < 0.05$)

The concentrations of primer were optimized based on the determined primer ratio. For *gadA/gadB* gene, the Ct values significantly decreased when primer concentrations increased. Ct values were very similar when concentration was varied from 400 nM to 900nM (Table

4.7). As a result, 600nM concentration was adopted. For *kan* gene, Ct values did not vary significantly under concentrations ranged from 200 nM to 600nM (Table 4.7). Thus, 200nM was applied in the following assays.

Table 4.7. Multiplex real- time PCR *gadA/gadB* and *kan* primers concentration optimization [a,b]

Primer concentration	Ct (*gadA/gadB*)	Ct (*kan*)
50nM	32.97 ± 1.29^{A}	20.28 ± 0.12^{A}
200nM	21.88 ± 0.38^{B}	17.89 ± 0.27^{B}
400nM	20.26 ± 0.68^{C}	17.83 ± 0.05^{BC}
600nM	19.43 ± 0.18^{C}	17.70 ± 0.03^{BC}
900nM	18.99 ± 0.07^{C}	17.56 ± 0.04^{C}

[a] Data are means of three replicates ± standard deviations.

[b] Tukey's pairwise comparisons was performed and values with different letters are significantly different ($p < 0.05$)

Finally, the probe concentrations of each gene were determined. For *gad*A/*gad*B genes, Ct values did not vary significantly under different concentrations indicating that higher probe concentration did not increase the reaction efficiency (Table 4.8). Therefore, the lowest concentration 50nM was employed. For *kan* genes, Ct values was significantly different when

probe concentration raised to 200 nM ($p<0.05$), but did not show differences between 50nM and 100nM (Table 4.8). Therefore, the lowest concentration 50nM was employed.

Table 4.8 Multiplex real- time PCR *gadA/gadB* and *kan* probes concentration optimization [a,b]

Probe concentration	Ct (*gadA/gadB*)	Ct (*kan*)
50nM	19.83 ± 0.03^{A}	18.74 ± 0.10^{A}
100nM	19.16 ± 0.04^{A}	18.86 ± 0.12^{A}
200nM	19.20 ± 0.05^{A}	19.96 ± 0.1^{B}
250nM	19.20 ± 0.06^{A}	19.96 ± 0.21^{B}

[a] Data are means of three replicates ± standard deviations

[b] Tukey's pairwise comparisons was performed and values with different letters are significantly different ($p< 0.05$)

The optimized multiplex PCR reaction efficiencies are 96.86% and 98.03% for *gadA/gadB* and *kan*, respectively (Fig 4.8). The R^2 for *gadA/gadB* and *kan* are 0.999 and 0.998, respectively, indicating that the multiplex real-time PCR reaction of *gad*A/*gad*B and *kan* is feasible and the following assays can be applied.

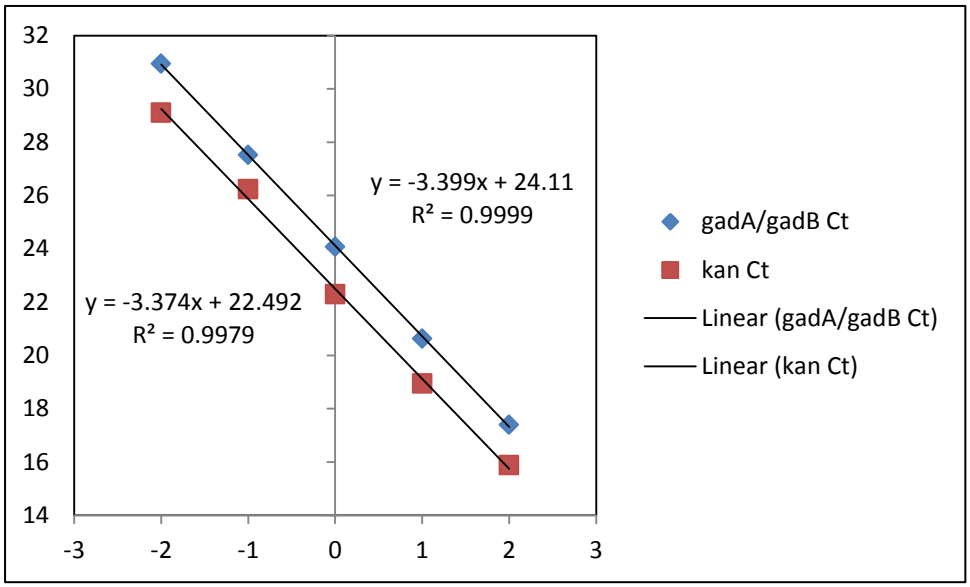


Fig 4.8 Standard curve for Multiplex real- time PCR of gene *gadA/gadB* and *kan*

4.4.4 Comparison of plating and real-time PCR

After establishing the culturing and real- time PCR methods, fresh manure, lagoon (a way of manure storage) and MAD effluent samples were tested to evaluate the efficiency of two methods. The DNA based molecular method detected higher value of *E.coli* numbers in fresh manure than culturing method (Figure 4.9). The error bars which represent standard deviations of triplication measurement indicate that culturing method has much larger within sample variation than molecular method.

E.coli numbers in sediment tanks samples were similar regardless of method used (Figure 4.10) between samples. Manure at the bottom of the tank (about 2m) had a slightly higher *E.coli* number than liquid collected at the surface. As in the fresh manure data, the standard deviations of culturing method were significantly larger than the molecular based method.

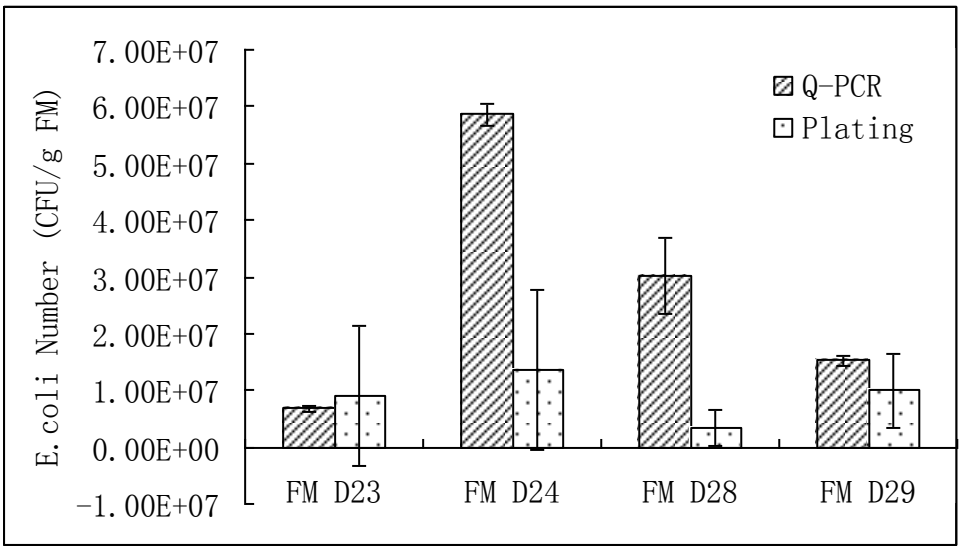


Fig 4.9. Comparison of plating and real- time PCR for fresh manure samples.
[a] Data are means of three replicates and error bars show standard deviations, p=0.157 by ANOVA test.

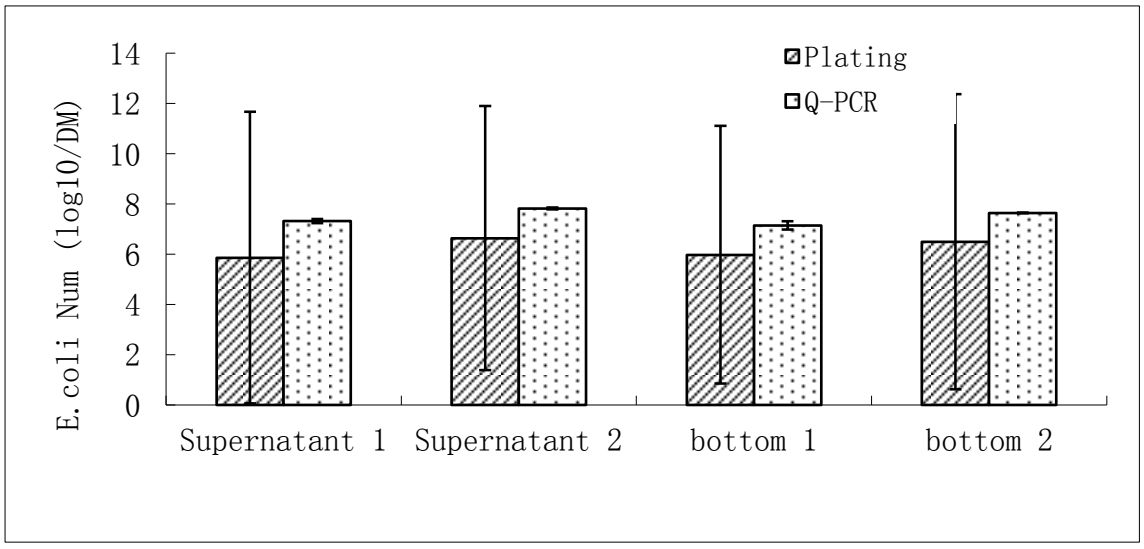


Figure 4.10 Comparison of plating and real- time PCR for sediment tank samples. Supernatant represent samples taken from the surface of the tank and bottom (2m depth) represents samples taken from the bottom of the tank. Data are means of three replicates and error bars show standard deviations.

E.coli numbers obtained by real- time PCR in MAD effluent samples were all larger than the plating number, although variability between the two methods was smaller with these samples.

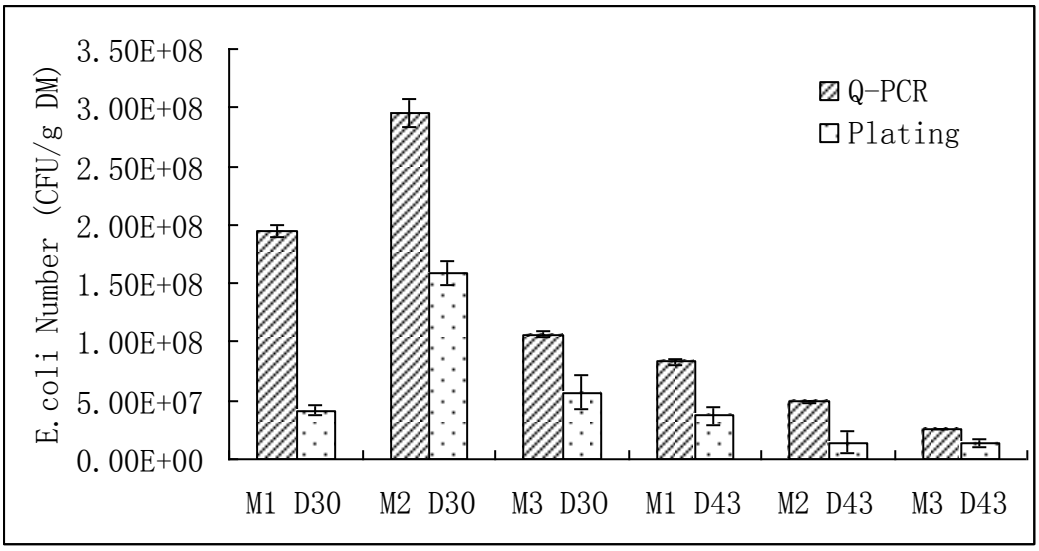


Fig 4.11. Comparison of plating and Q-PCR for anaerobic digestion samples
[a] Data are means of three replicates and error bars show standard deviations, p=0.157 by ANOVA test.

4.5. Discussion

Both the standard method EPA 1603 and Weaver's paper does not involve sonication step. In preliminary tests, *E.coli* numbers by unmodified plating method were always much lower than real- time PCR numbers (Fig 4.5). Based on the data, we established a hypothesis (section 4.2) that wild type bacteria including *E.coli* tends to grow on surface and form biofilm. Dairy manure has high contents of fiber and particles which provides advantages for attached growth. In this case, a piece of biofilm or one small particle that contains large number of *E.coli* may only produce one colony on plates and therefore is taken as

representing one cell. This phenomenon would cause underestimation of *E.coli* real number in manure samples. Sonication shakes the attached cells off the supporting surface and evenly distribute to solution. The data presents in Table 4.3 confirmed our hypothesis; *E.coli* cell number increased about 50% after sonication. Therefore, for high solid content samples, it is necessary to introduce sonication for even distribution.

*gad*A/*gad*B both encode an enzyme called Glutamate decarboxylase (GAD). GAD catalyzes the decarboxylation of glutamate to GABA and CO_2, and the reaction is shown as below.

$$HOOC\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH \rightarrow CO_2 + HOOC\text{-}CH_2\text{-}CH_2\text{-}CH_2NH_2 \qquad (5)$$

Although APHA recommends the other enzyme β-glucuronidase (GUD) as identical marker for *E.coli*, and EPA standard method 1603 also uses GUD as marker, research by Grant et al found that GAD is more specific for *E.coli* identification than the GUD especially for pathogenic strains such as *E.coli* O157:H7. Moreover, GAD is encoded by both *gadA* and *gadB*, for wild type *E.coli* the chance of losing both genes is rare. Thus, GAD is more suitable for molecular based detection.

Because of its high solid content character, dairy manure would contain small biofilm formed on fiber or particle surface and they could block the reagents from cell lysis and entrap the DNA. The nucleic acid extraction efficiencies and final yield vary considerably between different methods and type of environment samples. This makes the comparison of absolute number controversial. (Girones *et al.*, 2010)

Furthermore, the possible presence of inhibitors such as humic acid (a common soil supplement in agriculture) to the latter molecular assays could also limit the analysis of environment samples (Hu *et al.*, 2010; Techer *et al.*, 2010). Whether the level of inhibitors would affect the reaction efficiency of real- time PCR is also unknown. Therefore, it is necessary to calibrate the amplification efficiency between the environmental DNA and pure culture cell DNA which generate standard curve.(Smith and Osborn, 2009)

A DNA-based quantitative PCR method has been well-developed for municipal bio solid by Chen et al (Chen *et al.*, 2006). In this paper, a known DNA template was mixed with the solid and extracted together. However, the lysis efficiency of cells is still underestimated. To overcome these limitations, a control strain which do not contain *E.coli* specific target gene was constructed and used as an internal control for detection efficiency.

The efficiencies varied from 2% to 54%, even when the samples are from the same batch (Table 4.9). This table indicates that the detection efficiency would change even by the same DNA extraction method due to the complex manure matrix, the internal control for monitoring DNA extraction efficiency, inhibitor limitation and reaction efficiency is require to precisely measure the actual *E.coli* number.

Table 4.9 Dairy manure and anaerobic digestion samples' detection efficiency

Sample	FM[a] D23	FM D24	FM D28	FM D29		
Detection efficiency (%)	38.83	30.02	1.15	17.16		
Sample	M1[b] D30	M2 D30	M3 D30	M1 D43	M2 D43	M3 D43
Detection	62.09	92.06	52.58	54.57	47.86	2.00

efficiency (%)

[a] FM refers to fresh manure

[b] M refers to MAD samples, 1,2 and 3 represent the triplication

E.coli numbers quantified by real time PCR are always higher than plating number (Figure 4.9, 4.10, 4.11). Several reasons could contribute to this phenomenon: first, pathogen at a state called "viable but non-culturable" (VBNC) are undetectable by plating (Dinu *et al.*, 2009), whereas, other researchers proved that molecular based measurements were able to enumerate *E.coli* at VBNC state (Burtscher and Wuertz, 2003). Second, the high content fiber in dairy manure benefits the formation of biofilm and leads to underestimation of quantification by plating. Although sonication is employed to avoid the situation, it is not ensured that the sonication could release all cells into suspension. Third, since DNA extraction would extract the entire available DNA in samples, it is possible that cell debris in environment would also been extracted and quantified.

The error bar which present the standard deviation of triplication measurement is large for culturing data. In some cases, the standard deviation was even larger than the mean value. Dairy waste is not a homogeneous material so the fiber content may vary from aliquot to aliquot. Although the sonication step would shake attached cells off from fiber surface, the uneven distribution of fiber during serial had already caused the variation.

Molecular techniques such as quantitative PCR use specific primers and probes, target at unique gene region. They are highly sensitive, specific and fast tools for detecting and quantifying various pathogens in water, food and environment. Nevertheless, whether these techniques could replace the current conventional methods is still questionable. The detection

of genomes, unlike the traditional culture- based methods, could not distinguish between viable and non- viable organisms. Although some developed approach has been applied to discriminate damaged pathogen or naked nucleic acid, whether these methods could be applied to manure. What's more, molecular based method could not provide information about the infectivity of the pathogen or the level of risk for the population (Girones *et al.*, 2010).

4.6.Conclusion

In this study, a molecular based *E.coli* identification and quantification method is established. The traditional plating method is also modified and optimized in the research. Both methods were then applied to analyze fresh manure, lagoon, and anaerobic digestion samples. Several conclusions can be drawn: first, Duo to the high content of fiber in fresh manure, lagoon and anaerobic disgestion samples, it is necessary to sonicate diluted suspensions before filtration and plating; second, molecular based method could detect more *E.coli* cells in the samples than plating based method, because molecular based method could detect trapped cells and VBNC state cells while plating cannot; last, plating method would bring larger standard deviation than molecular method because of the uneven distributed fiber in samples.

Chapter 5 Fate of Pathogens during the anaerobic digestion treatment of dairy manure

5.1 Introduction

A variety of pathogenic microorganisms may be present in animal manure and slurries, including bacteria such as *Escherichia coli* (including O157:H7), *Salmonella* species, *Campylobacter jejuni* and *Listeria monocytogens*; parasites such as *Crytposporidium parvum* and *Giardia* species; and viruses such as enteroviruses (Bicudo and Goyal, 2003). Applying manure or slurry directly to the land can contribute to pathogen contamination of land, freshwater and groundwater (Aitken et al., 2007; Effenberger et al., 2003).

Different physical and chemical treatment has been applied for pathogen removal. Thermal treatment of manure in a pilot scale plant has been intensively studied for virus inactivation (Turner and Williams, 1999; Turner et al., 2000; Turner et al., 1999). Subsequently, studies of combination of chemical and thermal treatment were conducted in the same pilot scale plant to evaluate virus inactivation in pig slurry. It was found that virus and bacteria was inactivated to below detectable level at alkaline pH levels with the temperature of 50-55 ^{o}C; in acidified slurry (pH 6.4), the inactivation temperature would be 55-60 ^{o}C, indicating alkaline environment could enhance the pathogen removal efficiency (Turner and Williams, 1999; Turner *et al.*, 1999). As a powerful oxidizing agent, ozone was also reported as a very effective chemical for pathogen removal. It was studied that when high concentration of ozone was bubbled directly into fresh swine manure in a stirred batch reactor, the *E.coli* number reduced by more than 3 log units (Watkins B.D, et al 1996). Based

on oligodynamic (the inhibiting or killing of microorganisms by use of very small amounts of a chemical substance) actions, electrolytic treatment may also be able to kill some microorganisms by electrolysis. For example, electrolytic treatment combined with aeration of air reduced fecal coliforms in cattle slurry (Skjelhaugen and Donantoni, 1998).

Various biological treatments also have beneficial effect on pathogen removal. . Anaerobic lagoons are simple systems that can be very effective in decreasing organic matter and nutrient if properly designed and operated. It was studied that most pathogens would be reduced if enough time was given (Hill and Sobsey, 1998). Aeration (purging air into the reactor from the bottom) causes a pronounced effect on pathogen inactivation rate in cattle and pig slurry (Bicudo *et al.*, 1999). A decrease of one log unit of pathogen number was obtained by aeration within 2-4 days, whereas the same decrease was obtained within 200 days without aeration. Sequencing batch activated sludge reactor could also reduce fecal coliform numbers by 1-3 log units by intermittent aeration (Bicudo and Svoboda, 1995). Anaerobic digestion (AD) is another effective way for pathogen removal. Besides, AD also offers a number of other benefit including odor control, reduction in greenhouse gas emission and beneficial byproduct.

The objective of this work is to use the molecular-based method developed in previous work to study the pathogen fates in various anaerobic digestion processes.

5.2. Methods and materials

5.2.1 Fresh Manure samples preparation

The manure samples preparation was conducted as described in section 3.3.1

5.2.2 Anaerobic Digestion processes

Three anaerobic digestion processes were used: mesophilic anaerobic digestion (MAD), thermophilic anaerobic digestion (TAD) and temperature-phase anaerobic digestion (TPAD). Figure 5.1 illustrates the operational conditions of the three digestion processes. For MAD, the raw manure was first diluted with tap water to reduce the solid content to 1% (w/w) with a total volume of slurry being 500 ml. The slurries were then sealed in a 1 L glass bottle; nitrogen gas was used to purge the reactor to remove oxygen. The glass bottle was then incubated in shaker at 37 ^{o}C. The hydraulic retention time of the digester was 22 days; with 22.7 ml slurry was withdrawn from the digester, while the same volume of fresh manure slurry was fed to the digester on a daily basis.

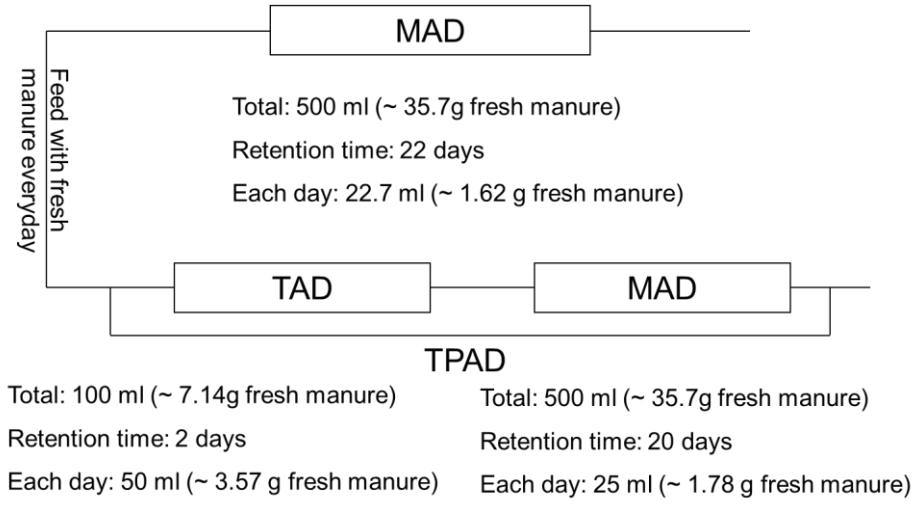


Figure 5.1 Scheme of anaerobic digestion operational conditions.

For TAD, the raw manure was first diluted with tap water to reduce the solid content to 1% (w/w) with a total volume of 100ml slurry. The slurries were then sealed in a 250ml serum bottles and flushed with N_2 gas for 1-2 min to create anaerobic conditions. The bottles were then incubated in an orbital shaker at 55^{o}C with continuous shaking (170 rpm). The hydraulic retention time of TAD is 2 days and 50 ml slurry was withdrawn while same volume of 1% (w/w) fresh manure was fed on a daily basis.

As shown in Figure 5.1, TPAD is the combination of TAD and MAD. The raw manure was first diluted with tap water, solid content was 1% (w/w), the total volume of TAD phase was 100ml, and the total volume of MAD phase was 500ml. Slurries were sealed in 250ml and 1L glass bottles, respectively. Nitrogen gas was implanted to eliminate oxygen. The operation temperature of TAD is 55 ^{o}C and the operation temperature for MAD phase is 37 ^{o}C. The retention time for TAD and TPAD were 2 days and 20 days, respectively. The TAD was fed with fresh manure every day and the MAD phase was fed with the effluent of TAD.

Total solid (TS) and volatile solid (VS), were determined according to standard methods (APHA, 1995), bio- methane was estimated by mixing the biogas with 1N NaOH solution and recording the gas volume after CO_2 absorption..*E.coli* numbers in fresh manure, MAD, TAD and TPAD effluents were determined by both culture-based method and molecular based method, the detailed information about procedures are described below.

5.2.3 Anaerobic digestion influents and effluents sampling schedule

MAD, TAD and TPAD was fed with 1% (w/w) fresh manure and withdrawn with same volume of effluents on a daily basis. The sampling started from day 22 when biogas production reached stable status till day 44 when anaerobic digesters were ceased. Effluents of digesters were sampled at different days for easy preparation. MAD effluents were collected on day 22, day 27, day 30, day 43 and day 44; TAD effluents were collected on day 23, day 28 and day 34; TPAD effluents were collected on day 24, day 29, day 34 and day 36. These sample dates were staggered to accommodate the time needed for analysis. Influent samples were collected on each sampling day regardless the type of digester. The collected influents and effluents were followed with TS and VS measurement and *E.coli* quantification by both plating and real-time PCR.

5.2.4 Enumeration of *E. coli* using cultural-based methods

The *E.coli* number in the samples was determined by U.S. Environmental Protection Agency (USEPA) Approved Method #1603. Although the method has been successfully applied to manure samples by Weaver et al (Weaver *et al.*, 2005), its accuracy and precision remains controversial. To prove our hypothesis as described in section 4.2, ultra-sonication was employed for shaking cells off from solid surface. Different treatments regarding the sonication time were tested, and the final treatment was described as below. The materials and protocol from Difco Laboratories (Detroit, MI) were used to implement the EPA 1603 methods. In brief, 1 g manure sample was resuspended in phosphate buffer solution

(NaH_2PO_4 0.58g/L, Na_2HPO_4 2.5g/L and NaCl 8.5g/L, pH 7.2) and stirred. The mixture was then diluted in serially with phosphate buffer solution. The final diluted solution was sonicated (VWR symphony Ultrasonic cleaner) for 5 minutes to improve distribution of *E.coli*, and then filtered through a 0.45 μm membrane (mixed cellulose esters, Millipore). After filtration, the membrane was placed on modified membrane-thermo tolerant *Escherichia coli* agar plates (modified mTEC). The plates were first incubated at 35 ^{o}C for 2 hours, and then at 44.5 ^{o}C for 22-26 hours. After incubation, the red or magenta colonies that appeared were counted.

5.2.5 DNA extraction

The solvent-based extraction protocol was based on Fuentes' paper (Arbeli and Fuentes, 2007) with some modifications (Eric F. Johnson and Biswarup Mukhopadhyay, unpublished information). In brief, 0.5 g of manure or slurry was weighed and 3 mL of lysis buffer (100mM Tris–HCl, 100mM Sodium EDTA, 100mM Na_2HPO_4, 1.5M NaCl, and 1% hexadecylmethylammonium bromide (CTAB, pH= 8)) was added to the samples. Samples were vortexed with 1 g of 1 mm glass beads and 50 μl of lysozyme (100 mg/ml) for 15 sec. The mixture was incubated at 37 ^{o}C for 30 min, then protease K and DTT was added and the mixture was incubated at 37 ^{o}C for another 30 min. The mixture was mixed with 400 μl 20% SDS and incubated at 65 ^{o}C for 1 hr. After incubation, the samples were centrifuged at 6000 ×g for 5 min and the supernatant was placed in a clean tube. The remaining pellets were re-extracted by lysis buffer and 20% SDS for 10 min, and the mixture was centrifuged at

6000 ×g for 5 min. Supernatants of the two extractions were combined and subjected to further PCI (Phenol/choroloform/isoamyl alcohol) and CI (choroloform/isoamyl alcohol) purification.

5.2.6 Enumeration of *E. coli* by real-time PCR

gadA and *gadB* has been identified as a suitable target in quantitative PCR experiments for estimating *E. coli* population density in environmental samples (Grant *et al.*, 2001). In our studies, the *gad*A and *gad*B genes were used as PCR targets in the *E.coli* detection. As metioned in section 4.2.3, the designed strain *E. coli* BW25113 Δ*gadA ⊿ gadB::kan* lacked those target genes so that it can be distinguished from the wild type *E.coli* presented in dairy manure and anaerobic digesters effluents. Meanwhile, the inserted kan gene would allow the quantification of added control strain numbers which help determining the detection efficiency (an co efficiency represents the loss of DNA during extraction and inhibition of PCR).

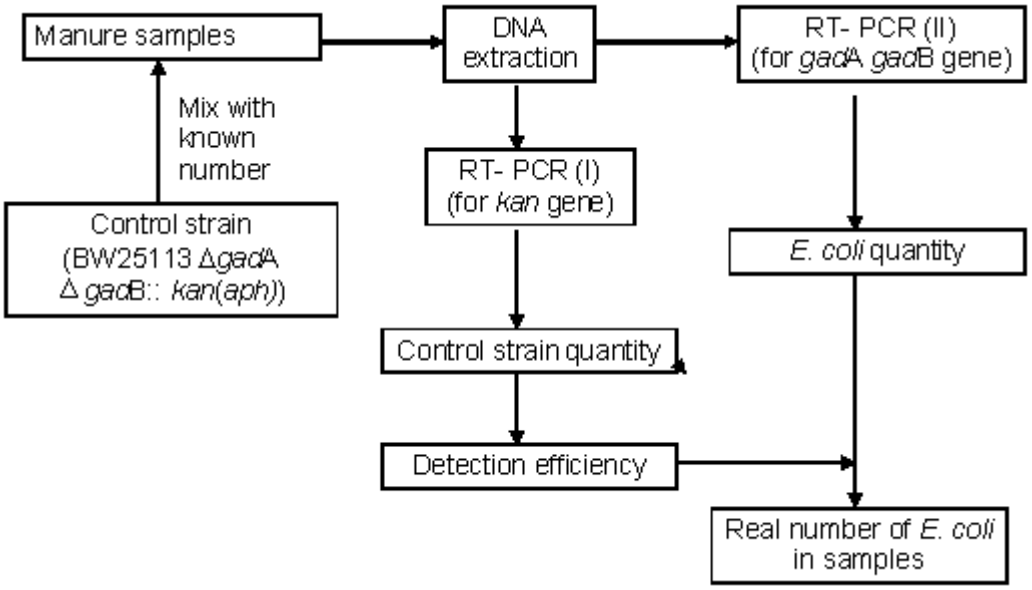


Fig 5.2. *E.coli* quantification method in dairy manure samples

Manure or anaerobic digesters effluent samples were mixed with pure control strain *E. coli* BW25113 Δ*gadA ⊿gadB::kan* (Fig 4.2*).* Subsequent DNA extraction was carried out by the method described in section 4.2.4. A multiplex real-time PCR was then performed to determine the Ct values (the number of cycles) of each gene, where the control (*E. coli* BW25113 Δ*gadA ⊿gadB::kan*) and the target organisms in the manure (*E. coli*) were detected and quantified simultaneously. From the Ct values for *kan* gene, the number of control cells added to the sample was calculated from a standard curve developed by pure culture of the strain. By comparing the calculated number with actual number of cells, detection efficiency (a co efficiency of both DNA extraction and PCR effectiveness) would be obtained. After the *E. coli* number was calculated from *gadA/gadB* Ct value with a standard curve for the pure culture of BW25113, the number was further corrected by the detection efficiency to determine the final value of real *E. coli* cells in samples.

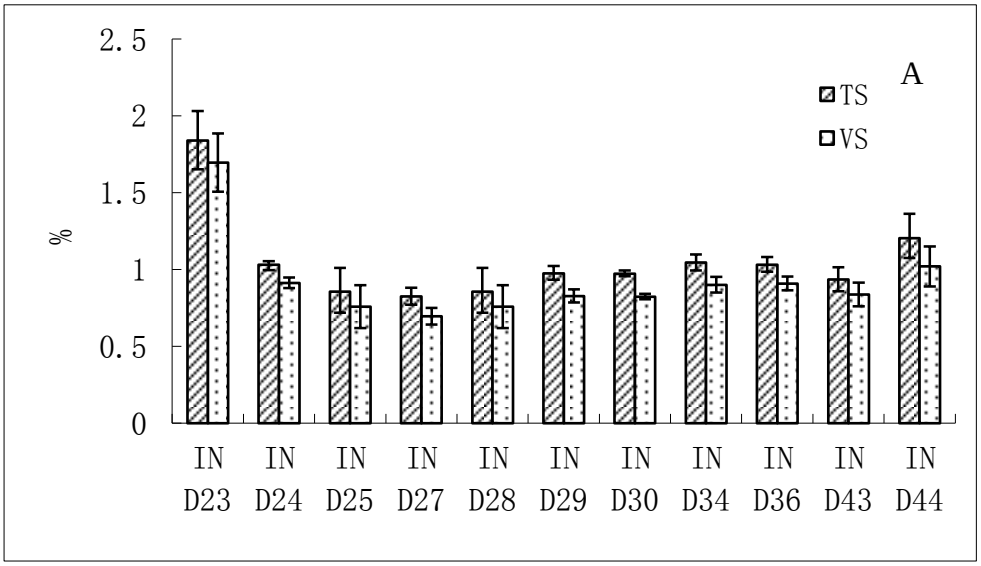
5.2.7 Statistical analysis

The mean values and standard variation of the experimental results was calculated by Microsoft excel 2003. Statistical significance of the experimental data was tested using one way analysis of variance (ANOVA) by JMP. Tukey's pairwise comparisons were used to test differences between treatments.

5.3 Result

5.3.1. Solid reduction and methane production of different anaerobic digestion processes

The three anaerobic digestion processes performed in this study were first evaluate by their solid reduction and methane prodiuction performances. Influent samples collected at day 23 had higher TS (1.84%) and VS (1.7%) than other samples ranged from 0.86% to 1.03% (Fig 5.3, 5.4).


A
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VS
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2
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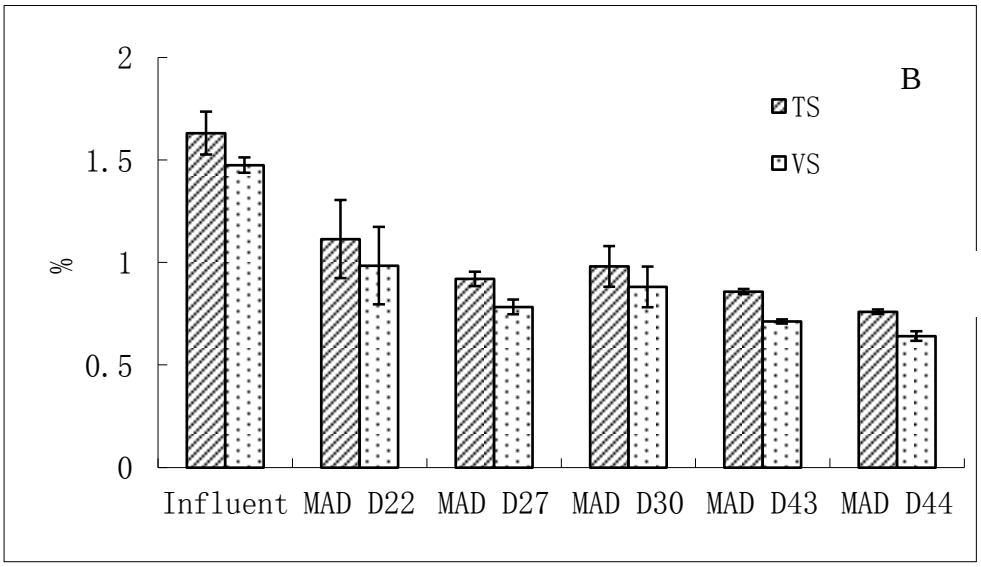
B
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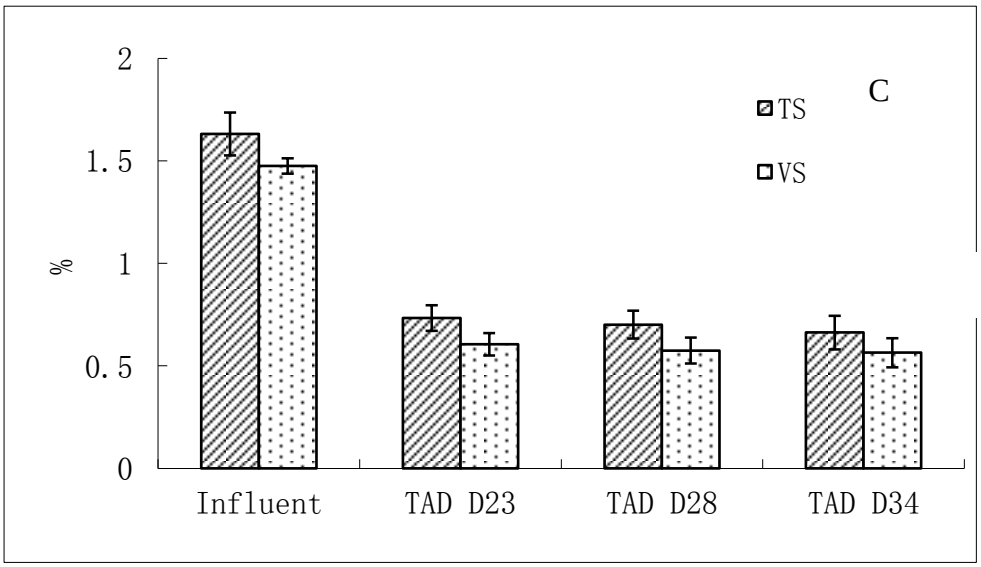
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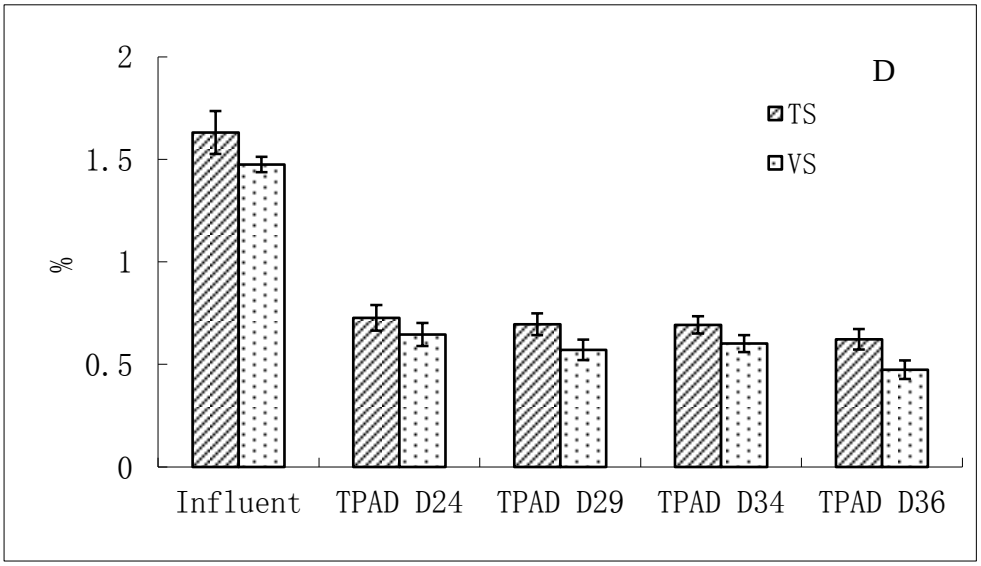

Figure 5.3 Total solid (TS) and volatile solid (VS) of influent, MAD, TAD and TPAD effluents. A: TS, VS of influent; B: TS, VS of MAD effluents; C: TS, VS of TAD effluents; D: TS, VS of TPAD effluents.

[a] Data are means of three replicates and error bars show standard deviations.

[b] IN refers to influent and D refers to sampling day.

All the MAD effluents had lower TS and VS than influents (Fig 5.3B). The values of TS and TS decreased slightly with time. Similar to MAD effluents, TS and VS content of TAD and TPAD effluent decreased regularly with time (Fig 5.3C, 5.3D). Compared to MAD and influent samples, TAD and TPAD have lower TS and VS values.

MAD had lower bio-methane production compared to TAD and TPAD. MAD had a relatively stable biogas production while the biogas production of TAD and TPAD fluctuated between 30 – 35 ml/VS per day and 40- 50ml/VS per day.

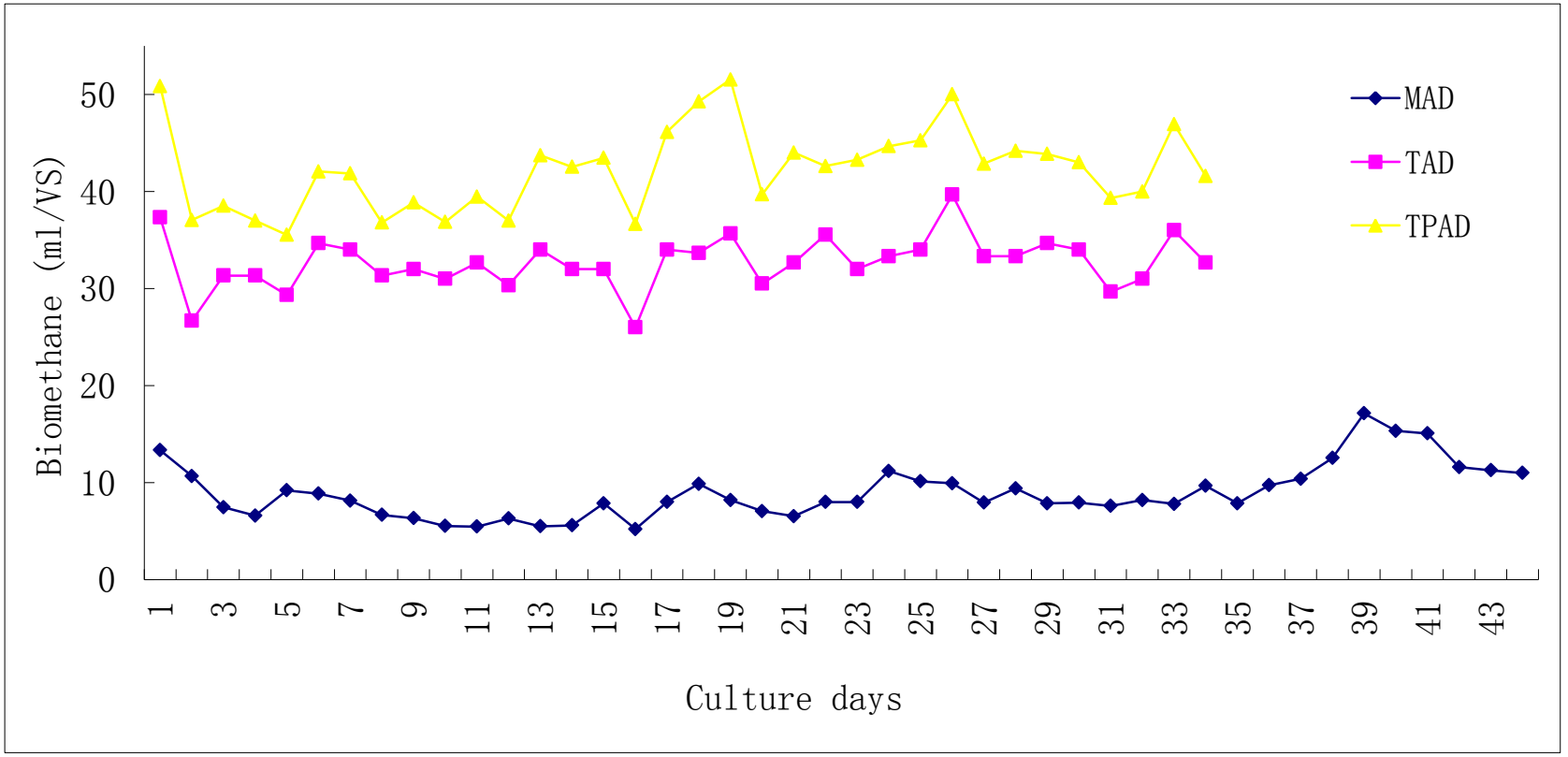

Figure 5.4 Bio-methane productions of MAD, TAD and TPAD. Data are means of three replicates

5.3.2 *E.coli* numbers of influent, mesophilic, themophilic and temperature- phase anaerobic digestion effluents

The *E.coli* numbers of influent, MAD, TAD and TPAD effluent were measured by both real-time PCR and plating methods. As shown in Figure 5.5, the *E.coli* numbers of influent samples varied each sampling day with the highest value of 1.34E+08 CFU/DM, and the lowest value of 6.75E+06 CFU/DM. Except for influent on day 23, the *E. coli* cell numbers determined by real- time PCR were higher than those of plating number ($p<0.05$) with the biggest difference of 1.24E+08 CFU/DM on day 28. Plating data had markedly larger error bars than real-time PCR data. On day 23, the standard deviation value was even larger than the mean values.

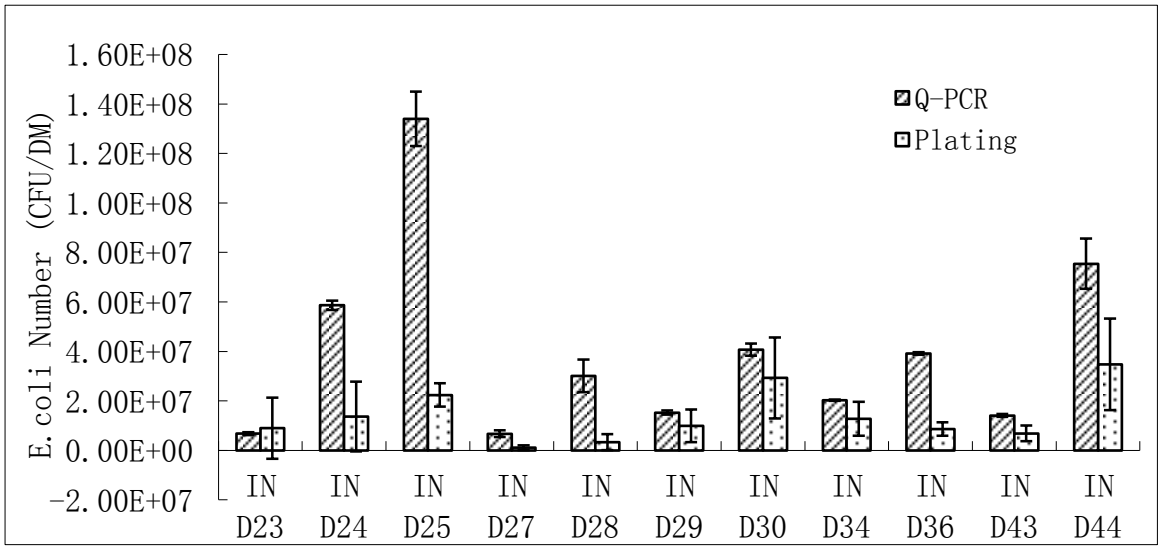

Figure 5.5 *E.coli* numbers of influents by both quantitative PCR and plating methods.
[a]Data are means of three replicates and error bars show standard deviations, $p<0.05$ by ANOVA test.
[b] IN refers to influent and D refers to sampling day

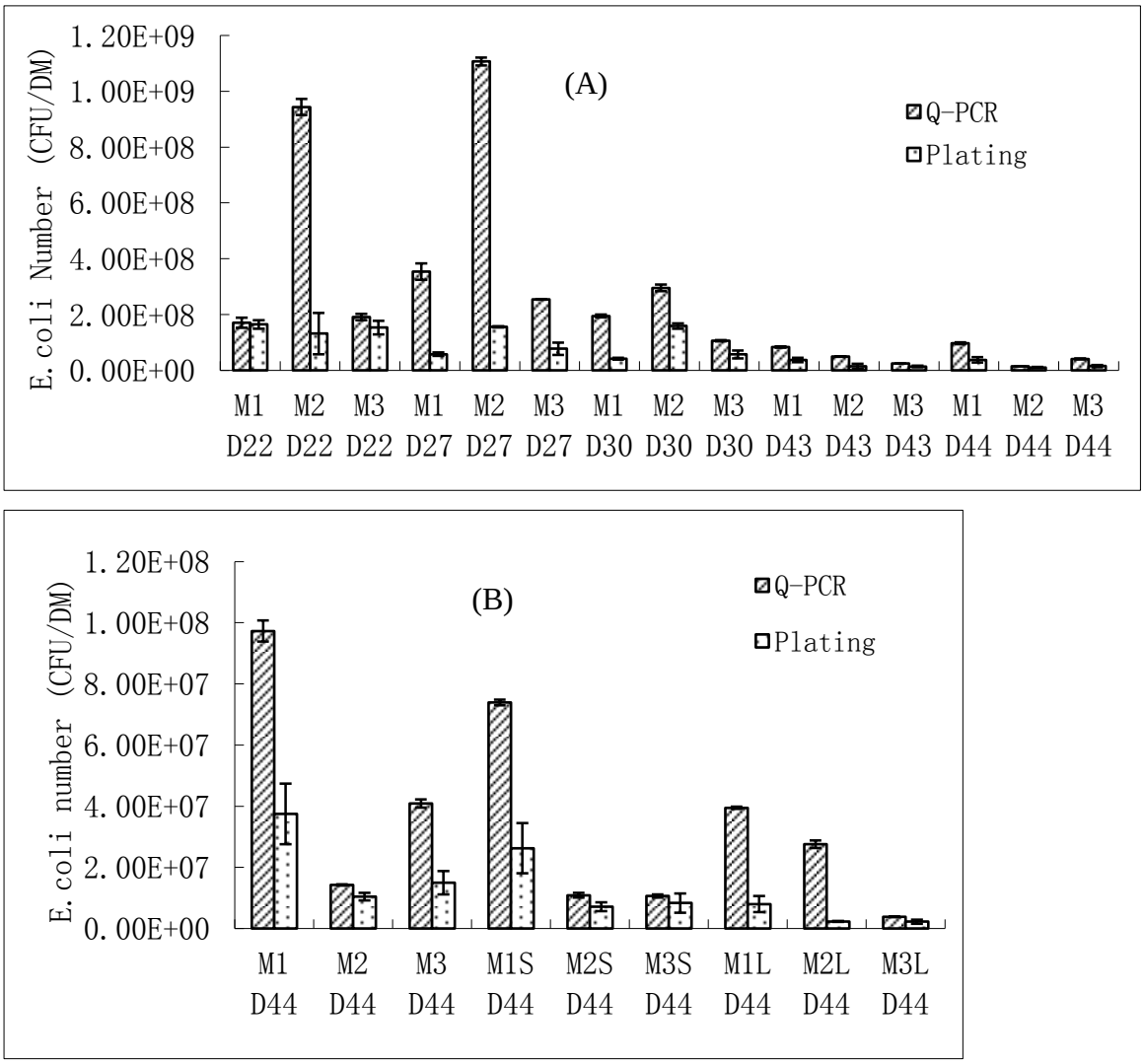

Figure 5.6 *E.coli* numbers of MAD effluents by both quantitative PCR and plating methods. A: MAD effluents collected at day 22, day 27, day 30 and day 43; B: MAD effluents collected at day 44, S represents solid phase and L represents liquid phase.

[a]Data are means of three replicates and error bars show standard deviations, $p<0.05$ by ANOVA test.

[b] M1, M2 and M3 are triplications of mesophilic anaerobic digesters and D refers to sampling day.

Similar as the influents data, real-time PCR detected more *E.coli* cells than plating method ($p<0.05$) in MAD effluents (Fig 5.6A). However, the standard deviation showed smaller difference between the two methods. The *E.coli* numbers gradually decreased across the period, and the lowest value was obtained on day 44. However, even the lowest number of *E.coli* for MAD efflunt was higher than influent *E.coli* numbers.

To study the fate of *E.coli* within the digester, both the liqid and solid phase of MAD effluents were measured. As can be seen from Figure 5.6B, more than 70% of the total *E.coli* were detected in the solid phase, and the rest were found in the liquid phase, indicating *E.coli* tend to land to solid surface in the digester.

TAD and TPAD effluent data had the similar trend as MAD effluent data with real-time PCR detecting higher number of *E.coli* than plating ($p<0.05$), and the E.coli numbers in effluent decreased with time during the digestion period. Compared to other digesters effluent, TAD efffluents had significantly lower *E.coli* numbers than others ($p<0.05$). More than 95% of the *E.coli* were removed in the digester, indicating that temperature is a key factor for pathogen removal. *E.coli* numbers in TPAD effluents started with more *E.coli* than was in the influent, and decreased wih time to the value that was at least 1 log number lower than influent values.

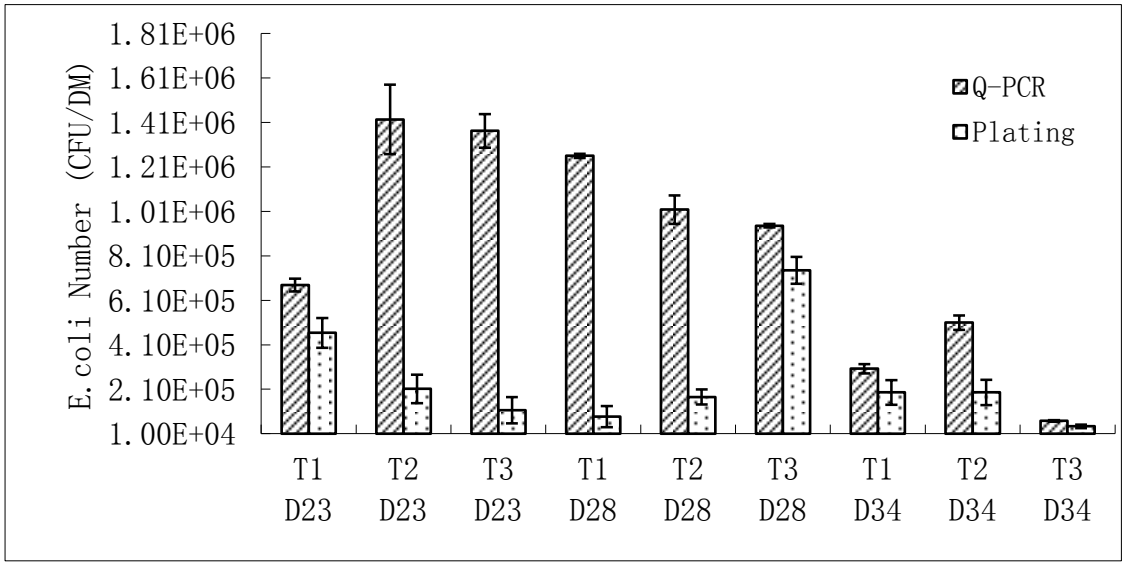

Figure 5.7 *E.coli* numbers of TAD effluents by both quantitative PCR and plating methods.
[a]Data are means of three replicates and error bars show standard deviations, $p<0.05$ by ANOVA test.
[b] T1, T2 and T3 are triplications of mesophilic anaerobic digesters and D refers to sampling day.

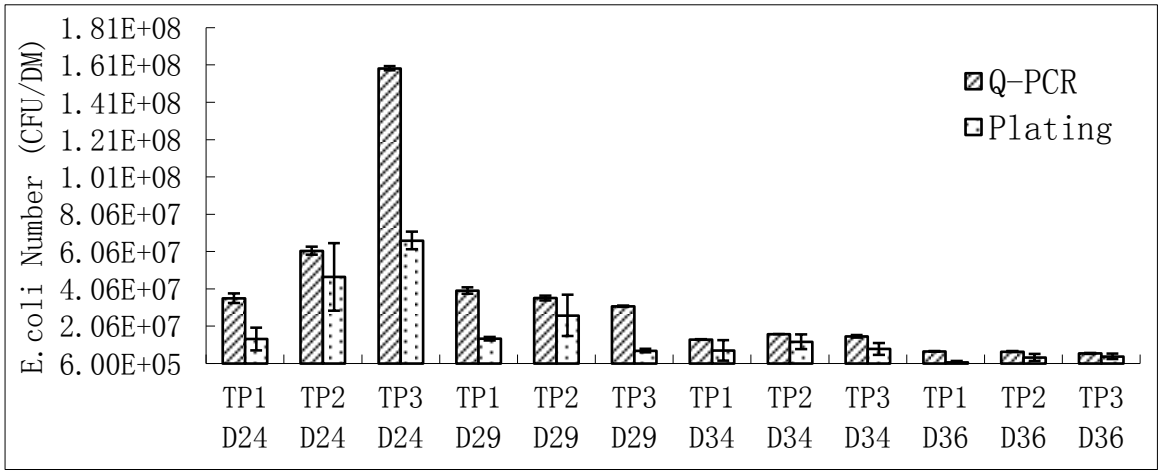

Figure 5.8 *E.coli* numbers of TPAD effluents by both quantitative PCR and plating methods.
[a]Data are means of three replicates and error bars show standard deviations, $p=0.198$ by ANOVA test.
[b] TP1, TP2 and TP3 are triplications of mesophilic anaerobic digesters and D refers to sampling day.

5.4 Discussion

Most of the *E.coli* was found in solid phase of the effluent samples, indicating that *E.coli*

tend to grow on solid surface in sediments (Fig 5.6B). This phenomenon may provide some explanations to two issues. The error bars of plating data were larger than the real time PCR data (Fig 5.6, 5.7, 5.8, 5.9). In the influent, the standard deviation was even larger than the mean value. Dairy manure is not homogeneous; the fiber content which represents the surface area for *E.coli* growth would vary from aliquot to aliquot, and the uneven distribution of fiber in aliquots during serial dilution of manure sample would cause large variation in measurement. In anaerobic digester effluents, available surface area was less because of the digestion of fiber and particles. Thus led to the more suspended *E.coli* in liquid, so the standard deviations of effluent samples were smaller than in influent samples

E.coli numbers detected by real time PCR were higher than plating number (Fig 5.6, 5.7, 5.8, 5.9). *E.coli* cells tend to bind to fiber or particles when in fresh manure or anaerobic digester. On a growth plate for *E.coli* quantification, however, one particle with one or more cells would only produce one colony resulting in underestimation of *E.coli* by plating method. Moreover, VBNC pathogen though they can cause infection in vitro, cannot be detected by standard culturing methods (Chen et al., 2006; Higgins et al., 2007; Makino et al., 2000). Meanwhile, it is also possible that in molecular based method, cell debris in samples was also been quantified and contributed as a part of the values.

TAD had much significantly smaller *E.coli* number than other digesters (Fig 5.7). Compared to influent data, more than 95% of the *E.coli* was removed from slurries. High temperature, by inactivating the protein on cell membranes, is an effective way to remove both pathogenic bacteria and viruses from the bio-solid (Turner and Williams, 1999; Turner

et al., 2000). Some pilot plants claimed an inactivation of all pathogens by heating bio-solids to 90 ^{o}C for 1-2 hours. This treatment is less effective in dairy manure due to its high content of fiber. The high solid content would form a large area of dead zone by affect the heat transform rate inside the plant, thus reducing the pathogen removal rate. In our case, lower temperature but longer retention time was employed, the longer retention time allowed improved heat transfer to slurries and thus inactivated pathogens.

It was found that *E.coli* number in MAD, TAD and TPAD effluents reduced over time. As a facultative microorganism, *E.coli* is able to grow both in aerobic or anaerobic conditions. Moreover, MAD and the mesophilc phase of TPAD at 37 ^{o}C is the best growing temperature for *E.coli*. The possible reason for *E.coli* reduction could be the competition from other anaerobic methanogenic microorganism. Anaerobic environment is favorable to those anaerobic organisms, so that better growth and greater consumption of nutrients led to the reduction of *E.coli* number.

Although TAD and TPAD are useful tools for pathogen removal, further studies of pathogen fate in different treatment systems are still needed. TAD could remove 95% of the *E.coli* in fresh manure, the absolute number of *E.coli* remaining large. The combination of other effective methods is required.

5.6 Conclusions

In this research, *E.coli* fate was studied in influents, mesophilic, themophilic and temperature- phase anaerobic digestion treatment systems. Several conclusions can be drawn:

first, *E.coli* numbers in mesophilic anaerobic digester were much higher than *E.coli* numbers in influent indicating anaerobic condition could not remove pathogen within short time. Second, more than 95% of the *E.coli* cells were removed in themophilic anaerobic digester. High temperature with proper hydraulic retention time is effective for pathogen removal. Third, temperature- phase anaerobic digester had higher *E.coli* numbers than influents at the beginning and the value reduced with time. At day 44, the *E.coli* number was much lower compared to influent numbers. Since TPAD produced the largest volume of biogas among MAD, TAD and TPAD, TPAD could also be considered as an effective treatment for dairy manure.

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