Use of Anaerobic Digestion to Destroy Biohazards and to Enhance Biogas Production

Background of the Invention

Many protein-based bio-hazardous materials constitute a major health problem world-wide. One of the major categories of such materials includes viruses.

For example, influenza virus is a member of the Orthomyxoviruses causing wide-spread infection in the human respiratory tract, but existing vaccines and drug therapy are of limited value. In a typical year, 20% of the human population is afflicted by the virus, resulting in 40,000 deaths. In one of the most devastating human catastrophes in history, at least 20 million people died worldwide during the 1918 Influenza A virus pandemic. The threat of a new influenza pandemic persists because existing vaccines or therapies are of limited value. In elderly the efficacy of vaccination is only about 40%. The existing vaccines have to be redesigned every year, because of genetic variation of the viral antigens, the Haemagglutinin HA and the Neuraminidase N. Four antiviral drugs have been approved in the United States for treatment and/or prophylaxis of Influenza. However, their use is limited because of severe side effects and the possible emergence of resistant viruses.

In the U.S., the major cause of diarrhea is virus infections, such as norovirus, rotavirus and other enteric viruses.

HIV (formally known as HTLV-III and lymphadenopathy-associated virus) is a retrovirus that is the cause of the disease known as AIDS (Acquired Immunodeficiency Syndrome), a syndrome where the immune system begins to fail, leading to many life-threatening opportunistic infections. HIV has been implicated as the primary cause of AIDS and can be transmitted via exposure to bodily fluids. In addition to percutaneous injury, contact with mucous membranes or non-intact skin with blood, fluids containing blood, tissue or other potentially infectious body fluids pose an infectious risk.

Many of these infectious viral agents, after coming into contact with certain biological materials, such materials become biohazard. Most (if not all) of these biohazard materials require a proper disposal.

Other protein-based bio-hazardous materials include prion, which may be present in so-called "specified risk materials (SRM)." Management of SRM, such as SRM from

cattle (as a potential BSE prion source), is still a global challenge. A cost-effective and environmentally responsible way to destroy prions and utilize decontaminated SRMs is highly desirable for the cattle industry.

BSE has been one of the biggest economic and social challenges to world's beef industry. In Canada alone, BSE caused a loss of over $6 billion since May of 2003. Transmissible spongiform encephalopathies (TSEs) form a group of fatal neurodegenerative disorders represented by Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI) in humans; and by scrapie, chronic wasting disease (CWD) and bovine spongiform encephalopathy (BSE) in animals (Collinge, 2001). Evidence accumulated during the major BSE epizootics in the UK (Belay *et al*, 2004) has confirmed a link between BSE and CJD. One critical step in preventing human infection is to eliminate the pathogen from the food chain and the environment, because transmission routes and mechanisms are not fully understood.

Prions are thought to be the pathogens causing TSEs. Prions, PrP^{sc}, are primarily comprised of a proteinase-K-resistant mis-folded isoform of the cellular prion protein PrP^{c} (Prusiner, 1998). Prions are resistant to inactivation methods usually effective against many microorganisms (Millson *et al*, 1976; Chatigny and Prusiner, 1979; and Taylor 1991, 2000). A number of studies have reported that chemical disinfection (Brown *et al*, 1982), autoclaving at 121°C for 1 hr (Brown *et al*, 1986, Taylor *et al*, 1997), exposure to 6 M Urea and 1 M NaOH (Brown *et al*, 1984, 1986), treatment with 1M NaSCN (Prusiner *et al*, 1981) and 0.5% hypochlorite (Brown *et al*, 1986), exposure to sodium hyperchlorite up to 14,000 ppm (Taylor, 1993), digestion with proteinase K (Kocisko *et al*, 1994; Caughey *et al*, 1997) and other newly identified proteases (McLeod *et al* , 2004; Langeveld *et al*, 2003) could not completely destroy the PrP^{sc}. Inactivation of PrP^{sc} in renderings has been evaluated in the UK and Europe (Taylor and Woodgate, 2003).

Enzymatic degradation of PrP^{sc} has also been studied as a means to achieve decontamination and reuse of contaminated equipment. For example, using the Sup35Nm-His6 recombinant prion protein to represent the BSE prion, Wang showed that surrogate BSE was selectively digested by subtilisin and keratinase but not by collagenase and elastases (Wang *et al*, 2005). Six strains of bacteria from 190 protease-secreting isolates were reported to produce proteases which exhibited digestive activities against PrP^{sc} (Myller-Hellwig, *et al*, 2006). Some thermostable proteases produced by the

bacteria degraded PrP^{sc} at high temperature and pH 10 (Hui *et al*, 2004, McLeod *et al*, 2004, Tsiroulnikov *et al*, 2004, Yoshioka *et al*).

So far, however, incineration is the only effective method to completely destroy prion. But incineration has certain undesirable ecological disadvantages, particularly energy consumption and green house gas emissions. For example, although the CFIA (Canadian Food and Inspection Agency) sanctions only incineration, alkaline hydrolysis and thermal-hydrolysis methods for the safe disposal of SRMs, incineration seems impractical for handling SRMs, especially in large scale, partly because of the industry's lack of capacity and the high associated costs. The limited capacity of existing incinerators and alkaline or thermal hydrolysis facilities, combined with the cost burden of carrying out these processes for destroying SRMs create onerous challenges to the livestock industry. It is estimated that 50,000 to 65,000 tones of SRMs are produced in Canada annually (Facklam, 2007). Incineration of SRMs consumes not only energy but also emits significant amounts of green house gas. In addition, end-products from these procedures are not useful for production of value-added byproducts.

Summary of the invention

One aspect of the invention provides a method for reducing the titer of a biohazard that may be present in a carrier material, comprising providing the carrier material to an anaerobic digestion (AD) reactor and maintaining the rate of biogas production substantially steady during the AD process.

In certain embodiments, the biohazard comprises hormones, antibodies, body fluids (*e.g.*, blood), viral pathogens, bacterial pathogens, and/or weed seeds. In other embodiments, the biohazard comprises prion. For example, the prion may be scrapie prion, CWD prion, or BSE prion. The prion may be resistant to proteinase K (PK) digestion.

In certain embodiments, the carrier material may be a protein-rich material. For example, the carrier material may be a specified risk material (SRM). The SRM may comprise CNS tissue (*e.g.*, brain, spinal cord, or fractions / homogenates / parts thereof).

As used herein, "protein-rich material" includes materials that are high (*e.g.*, 5-100% (w/w) protein, 10-50% protein, 15-30% protein, 20-25% protein) in protein content, which may be measured by various protein assays or nitrogen content assays known in the

art, such as the Kjeldahl method or derivative / improvements thereof, the enhanced Dumas method, methods using UV-visible spectroscopy, and other instrumental techniques that measures bulk physical properties, adsorption of radiation, and/or scattering of radiation, *etc.*

In certain embodiments, the nitrogen content of the added protein-rich material is about 5-15%, or about 10%.

In certain embodiments, the ratio of the added carrier material (as measured by volatile solid content) to the existing disgestate in the tank is no more than 1:1 (w/w). Volatile solid content can be measured by, for example, heating the sample to about 550°C and determining the weight of the volatile (lost) portion.

In certain embodiments, the AD reactor may be operated in batch mode. The batch mode may last less than about 0.5 hr, 1 hr, 2 hr, 5 hr, 10 hr, 24 hr, 2 days, 3, 4, 5, 6, 7, 10, 20, 30, 40, 50, or 60 days. For viral and bacterial agents, the batch mode generally lasts from less than about a few hours to several days (*e.g.*, 1-7 days), depending on temperature used. For especially stable agents, such as prion, the batch mode generally lasts less than about 30, 40, 50, or 60 days.

In other embodiments, it may be operated in semi-continuous mode, or continuous mode.

In certain embodiments, a carbon-rich material is provided semi-continuously to the AD reactor to maintain substantially steady biogas production. The carbon-rich material may comprise fresh plant residues or other easily digestible cellulose, although other materials that are not carbon-rich per se may also be present. In certain embodiments, the carbon-rich substrate is periodically added (about 1-3% (w/v) of) to the AD reactor.

In certain embodiments, the AD reactor contains an active inoculum of microorganisms at the beginning of the batch mode operation.

In certain embodiments, the AD process is carried out by a consortium of anaerobic microorganisms, such as psyclophilic microorganisms (*e.g.*, those with optimal growth conditions around 20°C or so), mesophilic microorganisms (*e.g.*, those with optimal growth conditions around 37°C or so), or thermophilic microorganisms (*e.g.*, those with optimal growth conditions above 45-48°C or so, such as 55°C, 60°C, 65°C).

In certain embodiments, the thermophilic microorganisms are acclimatized with substrates containing proteins with abundant β-sheets. This may be helpful for removing bio-hazard materials.

In certain embodiments, the thermophilic microorganisms are acclimatized by culturing with substrates containing amyloid substance at elevated temperature and extreme alkaline pH. The period can lasts, for example, for 3 months.

In certain embodiments, the method further comprises adding one or more supplemental nutrients selected from Ca, Fe, Ni, or Co.

In certain embodiments, the AD is carried out at about 20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C, or above.

In certain embodiments, 2 logs or more reduction of the titer of the biohazard (*e.g.*, prion) is achieved after about 60 days, 30 days, or even 18 days of anaerobic digestion.

In certain embodiments, 3 logs or more reduction of the titer of the biohazard (*e.g.*, prion) is achieved after about 20, 25, 30, 35, 40, 45, 50, 55, 60 or more days of anaerobic digestion.

In certain embodiments, 4 logs or more reduction of the titer of the biohazard (*e.g.*, prion) is achieved after about 30, 40, 50, 60, 70, 80, 90 or more days of anaerobic digestion.

In certain embodiments, 5, 6, 7, 8, or 9 logs of reduction of the titer of the biohazard (*e.g.*, bacterial or other non-prion biohazards) is achieved after about 10, 15, 20, 30, 40, 50, 60, 70, 80, 90 or more days of anaerobic digestion.

Another aspect of the invention provides a method for producing (high quality) biogas, comprising providing to an anaerobic digestion (AD) reactor a protein-rich feedstock, wherein the rate of biogas production is maintained substantially steady during the AD process.

In certain embodiments, the AD reactor is operated in batch mode.

In certain embodiments, the AD reactor contains an active inoculum of microorganisms at the beginning of the batch mode operation.

In certain embodiments, the batch mode lasts less than about 0.5 hr, 1 hr, 2 hr, 5 hr, 10 hr, 24 hr, 2 days, 3, 4, 5, 6, 7, 10, 20, 30, 40, 50, or 60 days. For many viral agents, the batch mode generally lasts less than about a few hours. For certain viral agents and many

bacterial agents, the batch mode generally lasts from less than about a few hours to several days (*e.g.*, 1-7 days). For especially stable agents, such as prion, the batch mode generally lasts less than about 30, 40, 50, or 60 days.

In certain embodiments, partly depending on the specific type of protein-based pathogens to be destroyed, the rate of biogas production peaks at about a few hours for many viral agents (*e.g.*, 0.5-5 hrs), or a few days for many bacterial agents (*e.g.*, 1, 2, 3, 4, 5, 6, or 7 days), or 5-10 days for many prions, after the beginning of the batch mode operation.

In certain embodiments, partly depending on the specific type of protein-based pathogens to be destroyed, a carbon-rich material is provided, semi-continuously to the AD reactor to maintain substantially steady biogas production. For example, the carbon-rich material may be provided once every about a few hours for many viral agents (*e.g.*, 0.5-5 hrs), or a few days for many bacterial agents (*e.g.*, 1, 2, 3, 4, 5, 6, or 7 days), or 5-10 days for many prions, after reaching peak biogas production.

In certain embodiments, the carbon-rich material comprises fresh plant residues, or other easily digestible cellulose.

In certain embodiments, the protein-rich feedstock comprises hormones, antibodies (*e.g.*, blood), body fluids, viral pathogens, or bacterial pathogens.

In certain embodiments, the protein-rich feedstock is a specified risk material (SRM).

In certain embodiments, the SRM comprises one or more prions or pathogens.

In certain embodiments, the prions comprise scrapie, CWD, and/or BSE prion.

In certain embodiments, the prions are resistant to proteinase K (PK) digestion.

In certain embodiments, the SRM comprises CNS tissue (*e.g.*, brain, spinal cord, or fractions / homogenates / parts thereof).

In certain embodiments, 2 logs or more reduction of the titer of the prions is achieved after about 60 days, 30 days, or even 18 days of anaerobic digestion. In other embodiments, 3 logs or more reduction of the titer of the prions is achieved after about 20, 25, 30, 35, 40, 45, 50, 55, 60 or more days of anaerobic digestion. In certain embodiments, 4 logs or more reduction of the titer of the bio-hazard is achieved after about 30, 40, 50, 60, 70, 80, 90 or more days of anaerobic digestion.

In certain embodiments, the AD is carried out at about 20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C, or above.

In certain embodiments, the bacteria carrying out the AD comprise a consortium of anaerobic microorganisms, such as psyclophilic microorganisms (*e.g.*, those with optimal growth conditions around 20°C or so), mesophilic microorganisms (*e.g.*, those with optimal growth conditions around 37°C or so), or thermophilic microorganisms (*e.g.*, those with optimal growth conditions above 45-48°C or so, such as 55°C, 60°C, 65°C).

In certain embodiments, the bacteria carrying out the AD is acclimatized with substrates containing proteins with abundant β-sheets.

In certain embodiments, the bacteria carrying out the AD is acclimatized by culturing with substrates containing amyloid substance at elevated temperature and extreme alkaline pH for 3 months.

In certain embodiments, the method further comprising adding one or more supplemental nutrients selected from Ca, Fe, Ni, or Co.

Another aspect of the invention provides a method for reducing the titer of a viral biohazard that may be present in a carrier material, comprising contacting the carrier material to a liquid portion of an anaerobic digestion (AD) digestate, preferably a thermophilic anaerobic digestion (TAD) digestate.

In certain embodiments, the contacting step is carried out at about 20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C.

It is contemplated that all embodiments described herein, including embodiments described separately under different aspects of the invention, can be combined with features in other embodiments whenever applicable.

Brief Description of the Drawings

Figure 1 shows results when scrapie-containing and normal sheep brain homogenates were spiked in TAD (thermophilic anaerobic digestion) digester, and incubated for a set period of time. The numbers 1 to 4 indicated different sampling times post digestively. The protein from the TAD-tissue mixtures at different time points was isolated, purified, and resolved by 12.5 % SDS-PAGE gel, and subjected to Western blotting detection with ECL substrate. Large amounts of prion proteins were recovered from TAD sludge before digestion (time 0). In contrast, none was found in TAD control

without the tissues. Cellular prion had disappeared at sampling time 1 (TAD-normal sheep brain mix), but scrapie was completely eliminated at sampling time 2 (TAD-scrapie mix). The 27 kDa protein marker indicates mobility of sheep cellular prion and scrapie prion.

Figure 2 demonstrates protein-load dependent methanation in the pilot study of scrapie inactivation during the course of TAD. TAD was set up with the same amount of the digestate containing different amounts of scrapie-infected sheep brain tissue and normal sheep brain tissue (in low dose and high dose, respectively). TAD alone was used as control. The highest volume of methane production was achieved in high-dose protein load groups (scrapie and normal sheep brain), and then in low-dose protein load groups (scrapie and normal sheep brain), in comparison with the control one. It indicates clearly that an increase of protein load at a given level in TAD enhances biogas production and CH_4/CO_2 ratio, thus increases fuel value of biogas.

Figure 3 shows assessment strategy for post-digest Scrapie prion samples in anaerobic digestion.

Figure 4 is a summary of time- and dose-dependent viral inactivation based on assessment of viral infection on cultured cells (cytopathic effect, CPE%).

Figure 5 demonstrates that Scrapie prion (S. prion) showed different degrees of reduction in the presence of absence of additional cellulosic substrates in TAD digestion processing at day 11, 18 and 26. The image was quantified using Alpha Innotech Image analyzer.

Detailed Description of the Invention

The invention is partly based on the discovery that peak destruction of certain biohazards in an anaerobic digestion (AD) system coincides with peak biogas production. Such biohazards may be present in a carrier material, and may include weed seeds, certain protein-rich pathogens or undesirable pertinacious materials (*e.g.*, hormones, antibodies, viral pathogens, body fluids (*e.g.*, blood), bacterial pathogens, *etc.*), or prions within a specified risk material (SRM). While not wishing to be bound by any particular theory, it is contemplated that at high biogas production rate, microbial activity is high or microbial growth rate is high, thus increasing the chance and/or rate of breaking down such biohazards.

The invention is also partly based on the discovery that certain small molecules within the anaerobic digestion (AD) system, especially the TAD system, may inactivate at least certain viral infectious agents. Thus such molecules, either purified or unpurified from the liquid anaerobic digestate, may be used to inactivate viral agents.

The invention is further based on the discovery that adding a carbohydrate-based substrate (such as cellulose or cellulose type material) periodically to the digester may accelerate or enhance the reduction of pathogen titer. The carbohydrate-based substrate may be added at a w/v percentage of about 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5%, 8%, 10%, 15%, or between any of the two referenced values (as measured by the weight (in gram) of the carbohydrate-based substrate over volume (in mL) of the digestate). One or more additions of the carbohydrate-based substrate may be made during the period of digestion. The intervals of adding the carbohydrate-based substrate may be substantially identical (*e.g.*, about 7-8 days between additions) or different. The timing of addition preferably substantially coincides with the biogas production rate, *e.g.*, just prior to or around the time peak biogas production is expected to dip.

Therefore, in one aspect, the invention provides a method for reducing the titer, amount, or effective concentration of a biohazard that may be present in a carrier material, comprising providing the carrier material to an anaerobic digestion (AD) reactor and maintaining the rate of biogas production substantially steady during the AD process after biogas production has reached a peak rate. The AD reactor may be operated in batch mode, semi-continuous mode, or continuous mode.

Rate of gas production may be measured in any of the industry standard methods, so long as a consistent method is used for monitoring gas production rate. Suitable methods include measuring gas pressure, gas flow rate, *etc.* Methane to carbon dioxide ratio may also be used for this purpose.

Almost any biohazard materials / agents can be the target of the subject method, including bacterial pathogens (*e.g.*, *E. coli*, *Salmonella*, *listeria*), viral pathogens (*e.g.*, HIV/AIDS, picornavirus such as foot-and-mouth disease virus (FMDV), equine infectious anemia virus, porcine reproductive and respiratory syndrome virus (PRRSV), also known as Blue-Ear Pig Disease, porcine circovirus type 2, bovine herpesvirus 1, Bovine Viral Diarrhea (BVD), Border Disease virus (in sheep), and swine fever virus), parasitic pathogens, prions, undesirable hormones, blood and other body fluids.

One particular type of biohazard, prion (scrapie prion, CWD prion, or BSE prion, *etc.*), is of particular interest. Such prion may be resistant to proteinase K (PK) digestion, and may be present in a protein-rich carrier material, such as a specified risk material (SRM).

As used herein, "specified risk material" is a general term referring to tissues originating from any animals of any age that potentially carry and/or transmit TSE prions (such as BSE, scrapie, CWD, CJD, *etc.*). These can include skull, trigeminal ganglia (nerves attached to brain and close to the skull exterior), brain, eye, spinal cord, CNS tissue, distal ileum (a part of the small intestine), dorsal root ganglia (nerves attached to the spinal cord and close to the vertebral column), tonsil, intestine, vertebral column, and other organs.

As used herein, "batch mode" refers to the situation where no liquid or solid material is removed from the reactor during the AD process. Preferably, the feedstock and other materials necessary for the AD process are provided to the reactor at the beginning of the batch mode operation. In certain embodiments, however, additional materials may be added to the reactor.

In contrast, in continuous mode or semi-continuous mode, solids and liquids are being continuously or periodically (respectively) removed from the AD reactor.

For example, the AD reactor may contain an active inoculum of microorganisms, *e.g.*, at the beginning of the batch mode operation. The active inoculum of microorganisms may be obtained from the previous batch of operation, with optional dilution to adjust the proper volume of the inoculum and the feedstock in the AD reactor. One associated advantage is that the microorganisms within the inoculum are already primed to produce biogas at optimal rate at the beginning of the operation, such that peak biogas production rate can be achieved in a relatively short period of time, *e.g.*, between about 5-10 days.

Due to the natural fluctuation of the biogas production rate, "substantially steady" means that the biogas production rate generally does not deviate from the average value by more than 50%, preferably no more than 40%, 30%, 20%, 10%, or less. Substantially steady gas production rate can be maintained by periodically adding to the anaerobic digestion reaction suitable amounts of additional substrates, preferably those do not

contain significant amount of pathogens to be destroyed (in the batch mode operation), at a time around the time point when peak or plateau gas production rate is about to decline.

In certain embodiments, a carbon-rich material may also be provided, semi-continuously to the AD reactor once every about 5-10 days after reaching peak biogas production, to maintain substantially steady biogas production. There are many suitable carbon-rich materials that can be used in the instant invention. In certain embodiments, the carbon-rich material may comprise fresh plant residues or other easily digestible cellulose.

The AD process is preferably carried out under thermophilic conditions, and such thermophilic anaerobic digestion (or "TAD") is shown to efficiently eliminate various biohazard materials such as SRMs (Specified Risk Materials), including materials containing various prion species. TAD provides several advantages for SRM destruction, including its thermo-effect, a hydraulic batch of homogeneous system with high pH, synergistic effects of enzymatic catalysis, volatile fatty acids, and/or biodegradation of anaerobic bacterial colonies. The TAD process also has the added advantage of allowing SRMs to be safely used as a biomass / feedstock source for the production of biogas and other byproducts.

Thus in certain embodiments, the temperature of the AD reactor is controlled at about 20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C, or above to facilitate a thermophilic anaerobic digestion (TAD) process. In certain preferred embodiments, the AD process is carried out by a consortium of thermophilic microorganisms, such as thermophilic bacteria or archaea.

Preferably, the starting pH of the TAD process is about 8.0, or about pH 7.5-8.5. pH regulating agents or buffers may be added to the reactor periodically, if necessary, to control the pH at a desired level throughout the AD process.

In certain situations, conventional TAD may or may not completely destroy prion or other biohazards / pathogens, possibly because of the lack of essential anaerobic bacterial colonies and enzymes required for the specific catalysis. Thus in certain situations, the anaerobic microorganisms may be acclimatized so that they are more adapted to destroying the intended target. For instance, in the case of prion, acclimatization can be done using substrates containing proteins with abundant β-sheets. For example, selected anaerobic digestates may be cultured with special substrates

containing amyloid substance at elevated temperature and extreme alkaline pH for about 3 months. Cultures using such acclimatized microorganisms may be further optimized by monitoring and adjusting biogas production profile, composition, and total ammonia nitrogen (TAN) to ensure that no inhibition of anaerobic digestion occurs. In certain embodiments, supplemental nutrients (such as Ca, Fe, Ni, or Co) may be added to increase efficient removal of propionate as volatile fatty acid (VFA).

Optionally, genetic evolution of anaerobic microorganism colonies during acclimatization can be analyzed with real-time PCR-based genotyping using specially designed primers and probes. Furthermore, decontamination capability of these acclimatized anaerobic microorganism batches can be tested and compared with conventional TAD in regards to the elimination rate of the prion.

Destruction of any types of viral pathogens may be effectuated by using the subject methods. Exemplary (non-limiting) viral pathogens (or bio-hazardous materials containing such viral pathogens) that may be destroyed using the subject methods include: influenza virus (orthomyxovirus), coronavirus, smallpox virus, cowpox virus, monkeypox virus, West Nile virus, vaccinia virus, respiratory syncytial virus, rhinovirus, arterivirus, filovirus, picorna virus, reovirus, retrovirus, pap ova virus, herpes virus, poxvirus, headman virus, atrocious, Coxsackie's virus, paramyxoviridae, orthomyxoviridae, echovirus, enterovirus, cardiovirus, togavirus, rhabdovirus, bunyavirus, arenavirus, bornavirus, adenovirus, parvovirus, flavivirus, norovirus, rotavirus, and other enteric viruses. Other viral pathogens include those detrimental to animal health, especially those found in and responsible for various viral diseases of the livestock animals. Such viruses may be present in disease tissues of livestock animals.

Destruction of any types of bacterial pathogens may be effectuated by using the subject methods. Exemplary (non-limiting) bacterial pathogens (or bio-hazardous materials containing such bacterial pathogens) that may be destroyed using the subject methods include: bacteria that cause intestine infection, such as *E. coli* (particularly enterotoxigenic *E. coli* and *E. coli* strain O157:H7), which bacteria cause stresses for municipal wastewater treatment; bacteria that cause food-related outbreaks of listerosis, such as *Listeria M.*; bacteria that cause bacterial enterocolitis, such as *Campylobacter jejuni*, *Salmonella EPEC*, and *Clostridium difficile*.

Destruction of any types of parasitic pathogens may be effectuated by using the subject methods. Exemplary (non-limiting) parasitic pathogens (or bio-hazardous materials containing such parasitic pathogens) that may be destroyed using the subject methods include: *Giardia lamblia and Crytosporidium*.

Fungal or yeast pathogens can also be eliminated by the subject method.

Any of the pathogen containing materials may be used in the methods of the instant application. For example, in certain hospitals (including vet hospitals) or healthcare facilities, patient (human or non-human animal) stools and/or body fluids (*e.g.*, blood) may be rich sources of viral, bacterial, and/or parasitic pathogens that should be decontaminated before releasing to the public water or waste disposal. Such bio-waste materials may be used as carrier materials for the methods of the invention.

Destruction of numerous types of prions may be effectuated by using the subject methods. As used herein, "prion" includes all infectious agents that cause various forms of *transmissible spongiform encephalopathies* (TSEs) in various mammals, including the scrapie prion of sheep and goats, the chronic wasting disease (CWD) prion of white-tailed deer, elk and mule deer, the BSE prion of cattle, the transmissible mink encephalopathy (TME) prion of mink, the feline spongiform encephalopathy (FSE) prion of cats, the exotic ungulate encephalopathy (EUE) prion of nyala, oryx and greater kudu, the spongiform encephalopathy prion of the ostrich, the Creutzfeldt-Jakob disease (CJD) and its varieties prion of human (such as iatrogenic Creutzfeldt-Jakob disease (iCJD), variant Creutzfeldt-Jakob disease (vCJD), familial Creutzfeldt-Jakob disease (fCJD), and sporadic Creutzfeldt-Jakob disease (sCJD), the Gerstmann-Sträussler-Scheinker (GSS) syndrome prion of human, the fatal familial insomnia (FFI) prion of human, and the kuru prion of human.

Certain fungal prion-like proteins may also be destroyed, if necessary, using the subject methods. These include: yeast prion (such as those found in *Saccharomyces cerevisiae*) and *Podospora anserina* prion.

The amount of prions or other biohazards / proteinaceous pathogens used in the subject method can also be adjusted. In certain embodiments, an equivalent of about 1-10 g, or about 2.5-5 g of prion-containing tissue homogenate is present in every about 60 to 75 ml of TAD-tissue mixture. For TAD-tissue mixture having protein load towards the

high end of the range, about 1 g of carbon-rich material (*e.g.*, cellulose) may be added according to the scheme described herein to every about 60-75 mL of TAD-tissue mixture.

In certain embodiments, the AD reactor contains at least about 5, 6, 7, 8, or 9% final total solid components.

In certain embodiments, the prion is resistant to proteinase K (PK) digestion.

In certain embodiments, the SRM comprises CNS tissue, such as tissues from brain, spinal cord, or fractions, homogenates, or parts thereof.

In certain embodiments, the batch mode operation lasts less than about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, or 120 days. At the end of the batch mode operation, the titer of the biohazard / prion is reduced by at least about 2, 3, or 4 logs. For example, in certain embodiments, 2 logs or more reduction of the titer of the biohazard / prion is achieved after about 60, 30, or even 18 days of anaerobic digestion. In certain other embodiments, 3 logs or more reduction of the titer of the bio-hazard / prion is achieved after about 20, 25, 30, 35, 40, 45, 50, 55, 60 or more days of thermophilic anaerobic digestion. In certain embodiments, 4 logs or more reduction of the titer of the bio-hazard / prion is achieved after about 30, 40, 50, 60, 70, 80, 90 or more days of thermophilic anaerobic digestion.

The invention is also partly based on the discovery that enhanced biogas (*e.g.*, methane or CH_4) production through anaerobic digestion can be achieved by using a protein-rich feedstock. Furthermore, biogas production may be further enhanced by semi-continuously providing a carbon-rich material, optionally together with additional protein-rich material, to the AD reactor in order to maintain the rate of biogas production substantially steady during the AD process, preferably also with high quality (*i.e.*, CH_4 higher than 50, 55, 60, 65, or 70%). While not wishing to be bound by any particular theory, the observed enhanced biogas production suggests that the AD process allows various microorganisms present in the AD bioreactor to breakdown the protein-rich feedstock to supply nitrogen and/or carbon for microbial growth, and ultimately methane production (*i.e.*, methanogenesis is highly efficient).

Thus in one aspect, the invention provides a method for producing biogas, preferably with higher fuel value and high quality, comprising providing to an anaerobic digestion (AD) reactor a protein-rich feedstock, wherein the rate of biogas production is maintained substantially steady during the AD process after a peak rate of biogas production is reached.

In certain embodiments, the AD reactor may be operated in batch mode. In other embodiments, the AD reactor may be operated in continuous or semi-continuous mode, with continuous or periodic addition and removal of solids / liquids from the reactor during the AD process.

Regardless of the operational mode, a carbon-rich material may be provided to the reactor during the AD process to sustain the peak rate of biogas production. For example, in the batch mode, the carbon-rich material may be semi-continuously or periodically provided to the AD reactor once every about 5-10 days after reaching peak biogas production rate, in order to maintain substantially steady biogas production. Such carbon-rich material may include fresh plant residues, or any other easily digestible cellulose. In continuous or semi-continuous mode operation, the carbon-rich material and optionally the protein-rich feedstock may be added either together or sequentially / alternatively to sustain steady state biogas production.

In certain embodiments, the batch mode operation may lasts less than about 30, 40, 50, 60, 70, 80, 90, 100, 110, or 120 days.

In certain embodiments, the biogas fuel value, as defined by the ratio of methane over CO_2, is roughly directly proportional to (or otherwise positively correlated with) the protein content in the feedstock. Under optimal conditions, protein degradation occurs rapidly during the first 5-10 days of the AD process. During this period, peak protein degradation coincides with peak biogas production rate.

Almost any protein-rich feedstock can be used for the instant invention. In certain embodiments, the protein-rich feedstock is a specified risk material (SRM). For example, the SRM may comprise one or more prions or pathogens. Such SRM may comprise CNS tissues (*e.g.*, brain, spinal cord, or fractions / homogenates / parts thereof). Prions may include scrapie, CWD, and/or BSE prions, *etc.* (*supra*). In certain embodiments, the prions are resistant to proteinase K (PK) digestion. Batch mode is preferred if SRM containing prion is used as the protein-rich feedstock.

In other embodiments, the protein-rich feedstock may comprise hormones, antibodies, viral pathogens, or bacterial pathogens, or any other proteinaceous substance.

Another aspect of the invention provides a protein extraction method to achieve the maximal recovery of prion proteins from anaerobic digestate. This method can be used, either alone or in conjunction with traditional biochemistry techniques (such as Western

blotting (WB) and any commercialized BSE-Scrapie Test kit, *etc.*), to examine and document the elimination rate of prions during and after the TAD process. Preferably, a series of positive controls may be included in the assay.

Another aspect of the invention provides a method to determine the presence and/or relative amount of residual prions in the post-digestion sample. The method may comprise one or more technologies useful for prion detection, or combinations thereof. In a preferred embodiment, as shown in Figure 3, post-digestion sample obtained at any given time points during the AD process may be subjected to successive rounds of analysis including EIA, Western Blotting (WB), iCAMP, and bioassay with transgenic mouse, progressing to the next level of (more sensitive but expensive / difficult / slower) analysis only when the previous level of (less sensitive but cheaper / easier / faster) analysis has failed to confirmed the absence of prion in the sample.

For example, if EIA is sufficient to detect the presence of prion, there will be no need to run more complicated assays to confirm the existence of prion. Only when EIA fails to detect prion would WB becomes necessary for the next level of analysis.

Similarly, in certain embodiments, when WB fails to detect prion after multiple tests, a highly sensitive detection method termed *in vitro* cyclic amplification of mis-folding protein (iCAMP) may be used to verify the absence of prion (thus the completion of prion destruction) in the TAD discharge. In certain embodiments, a repeatedly negative iCAMP sample can in turn be examined with, for example, a mouse-based bioassay to determine a biologically safe end-point of prion decontamination and to ensure zero-discharge of any prions into the environment.

These prion detection methods are well known in the art. See Groschup and Buschmann, Rodent Models for Prion Diseases, *Vet. Res.* 39: 32, 2008 (incorporated herein by reference). For example, there are several transgenic mouse models (*e.g.*, Tg 20) that can be used to verify the infectivity and transmission of prion / scrapie before and after AD inactivation. Most of such transgenic mice in prion research are knock-out mice, with their endogenous prion genes knocked out. They generally have increased susceptibility to prion pathogens, including prion pathogens from a different species. Symptoms of prion manifestation - pathological changes in the brain tissue of the affected animals - may be detected or verified using immuno-histochemistry methods, which is one of the most confirmative assays for diagnosis of prion diseases.

For example, US 2002-0004937 A1 describes such a transgenic mouse model for prion detection, comprising introducing a prion gene of an animal (*e.g.*, that of human, cattle, sheep, mouse, rat, hamster, mink, antelope, chimpanzee, gorilla, rhesus monkey, marmoset and squirrel monkey, *etc.*) into a mouse (preferably a mouse with its endogenous prion genes knocked out) to produce a prion gene modified mouse, and determining that the prion gene is aberrant when the prion gene modified mouse exhibits heart anomalies. Using this mouse, prion titer before and after AD may be measured by, for example, inoculating the transgenic mouse with a sample (before / after AD), and observing the presence of myocardial diseases in the prion gene modified mouse. Samples spiked with known titers of control prion of the same type may be used in the same experiments to quantitatively measure the prion titers before / after the TAD process of the invention.

More specifically, for use in the instant invention, samples obtained at, for example, day 30 or later (in which no prion proteins may be detectable by Western blot, or "WB"), and filtered for sterilization. Then about 50 to 80 µl (usually less than about 100 µl) of the sterilized sample is injected into the brain of a selected transgenic mouse under anesthesia, with undigested prion / scrapie as control in same strain of mice. Observation days is usually 100 to 150 days after inoculation. Earlier samples taken at earlier time points, such as day 18, 11 or even 6 (when WB may show detectable levels of prion / scrapie) may be used in parallel experiments to determine the time period where AD has substantially eliminated active prion in the sample. This type of bio-assay allows one to determine whether prion / scrapie has lost its infectivity, even though the prion protein itself may still be detectable by WB.

Most suitable transgenic mice are available in the art, including from commercial entities (*e.g.*, Jackson Laboratory).

In certain embodiments, the mechanism of prion inactivation and its conformational alteration in post-digest samples can be investigated using mass spectrometry and other proteomic tools (see Figure 3). This down-stream research can further expand the general knowledge of prion structure and its related pathogenesis, and provide collaborative opportunities for basic researchers to explore fundamental knowledge of prions and develop drugs for treatment of prion-associated diseases in humans (such as CJD).

Multiple advantages can be realized according to the instant invention. For example, prion (Scrapie or BSE, *etc.*) and its infectivity can be destroyed completely by the TAD within 30 days, 60 days, or 100 days. Meanwhile, protein-rich SRMs with disinfected prions, instead of being waste materials that require costly treatment for proper disposal, can be utilized by the TAD process to enhance fuel value of biogas in comparison to conventional anaerobic digestion. As a result, multiple social and economical benefits can be simultaneously achieved, including allowing the cattle industry to treat SRMs cost-effectively, meeting certain government mandates, protecting the environment from a possible contamination with prion pathogens, reducing the environmental footprint caused by the disposal of SRM treated by other methods, and at the meantime generating valuable biogas. Thus, thermophilic anaerobic digestion process may well eliminate prions in SRMs effectively via combined enzymatic catalysis and biological degradation by anaerobic bacterial colonies in the system, and turn the protein-rich SRMs into bioenergy and biofertilizers.

Examples

The invention having been generally described, the following section provides exemplary experimental designs that illustrate the general principle of the invention. The examples are for illustration purpose only, but not limiting in any respect.

In addition, although some examples below are based on prion proteins, other less stable protein-based bio-hazardous materials, including hormones, antibodies, viral pathogens, bacterial pathogens, and/or weed seeds, *etc.*, are expected to behave similarly, if not identical, in similar experiments.

Example 1 Thermophilic Anaerobic Digestion (TAD) Process Eliminates Scrapie Prion and Enhances Biogas Production

Scrapie prion, one of the very resistant prions to proteinase K (PK) digestion, was used as a model in this experiment to demonstrate the effectiveness of the TAD process for prion destruction.

High- (4 g) and low-dose (2 g) of scrapie brain homogenate (20%) were spiked into the lab scale TAD digesters, with temperature set at 55°C. Digestion was allowed to continue in batch mode for up to 90 days. About 5 mL of the digestate was taken from

experimental and control groups at day 0, 10, 30, 60, and 90 for assessing scrapie degradation. Scrapie (PrP^{sc}), obtained from the CFIA National Reference Lab, and cellular prion (PrP^{c}) were recovered from the digestate using a buffer containing 0.5 % SDS (recovery rate ~ 75 to 82%). Both cellular and scrapie prion were resolved in 12.5% SDS-PAGE gel and detected by immunoblotting using a monoclonal antibody (F89, Sigma). Biogas production was monitored regularly to assess activity of anaerobic bacteria and to evaluate effect of protein-rich substrate on biogas production using micro-gas chromatography (GC).

The results demonstrated that scrapie was degraded in a time-dependent manner. While the cellular prion had disappeared by about day 10, no scrapie band was observed at Day 30 in TAD digesters. It was estimated that at least about 2.0 logs or more reduction of scrapie was achieved in 30 days based on computer-assisted semi-quantitation of immunoblotting images. Meanwhile, biogas production and its fuel value (ratio of methane over CO_2) were enhanced significantly in protein-rich TAD. About 2.6-fold more methane was gained in high-dose protein (384.42 ± 6.54 NmL), and about 1.9-fold in low-dose protein TAD (284.39 ± 2.02 NmL) than that in TAD control without protein (145.93 ± 10.33 NmL) during 90 days' of AD digestion.

The data demonstrates that batch TAD can be effectively used as a biological and environment friendly method to decontaminate prion in SRM , and transform SRM from a biohazard into a safe feedstock for producing biogas and other value-added byproducts. This process not only reduces the environmental footprint of prions, but also generates economic benefit to both the cattle industry and local community.

Example 2 Efficacy and Kinetics of BSE Elimination in Batch-TAD under Optimal Conditions

Bovine brain tissue and other types of SRM tissues (such as spinal cord, lymph nodes or salivary glands) with confirmed BSE are obtained from the CFIA National BSE Reference Lab, and homogenized in phosphate buffered saline (PBS) on ice. A 20% brain homogenate alone or homogenate mixed with other tissues is spiked in diluted digestate (with final total solid of about 7%), which is obtained fresh from the IMUS™ demonstration plant in Vegreville, based on results of the studies described above. The whole procedure is carried out in a biosafety cabinet (class IIB) in a Biolevel III laboratory

(*e.g.*, in the Laboratory Building of Alberta Agriculture and Rural Development). Final content of the homogenate is about 2.5 and 5 grams (equivalent of fresh tissue) in TAD-tissue mixture in a low- and high-dose group, respectively. The mixture is then placed into a screw-capped, safety-coated glass bottle. Anaerobic digestion starts in an incubator with a temperature setting of 55°C and pH 8 with specific controls (see Tab. 1 for study design).

Table 1. Experimental Design

TAD-Tissue Mixture	Experiments				Controls			
	N (normal bovine brain)		B (BSE bovine brain)		DC (without brain)		IC (BSE brain and inactivated digest mixture)	
	N-low	N-high	B-low	B-high	DC-1	DC-2	IC-1	IC-2
Brain tissue containing BSE (gram)	2.5	5.0	2.5	5.0	-	-	2.5	5.0
Anaerobic Digestate	Same amount in each group (< 250 mL)							
Cellulose* (gram)		1		1		1		
Incubation @	55^0C							

*Cellulose is added to the digestion mixture as a carbon-rich material to provide extra carbohydrate and may boost digestive activity of the anaerobic bacteria.

Inactivated digestate control (IC) is designed to check whether there is degradation of BSE (B) in the silent digestion mixture without activity of live bacteria. Additional control group (N) includes normal bovine brain homogenate containing cellular prion. This allows checking elimination rate of cellular prion during the digestion process. A

correlation between the cellular and BSE prion predicts relative elimination rate of BSE prion during TAD process.

A similar experiment is also designed for TAD digesters containing bovine brain tissue and other types of SRM tissue mixtures in comparison with bovine brain alone.

Biogas production and composition is monitored with a pressure transducer and gas chromatography. The time course of BSE prion decontamination is assessed at different time points from Day 0 to 120. At each time point, total protein from samples is extracted, concentrated and purified using established methods, and subjected to analyses using SDS-PAGE, Western blotting (WB, Schaller *et al*, 1999, Stack, 2004) with a panel of specific monoclonal anti-prion antibodies recognizing different epitopes. Reduction of the BSE prion in post-digest samples is compared with a series of 10-fold dilutions of the same batch of BSE brain homogenate and the sample taken at time zero. The WB image is analyzed using a densitometry to semi-quantify the reduction of the BSE prion at different times and with different tissue mixtures. For all positive samples detected by WB, the samples are subjected to proteinase-K digestion to examine whether resistance of BSE prion has been altered during the TAD process.

Kinetics of BSE elimination in TAD is assessed using an equivalent amount of bovine brain homogenate containing cellular prion (PrP^c) as control. The rates of destruction of the bovine PrP^c and of the BSE prion are compared at different time points during the digestion process. A series of elimination percentiles of BSE at sequential time points provide relative kinetics of BSE destruction during the process.

Example 3 *In vitro* Cyclic Amplification Misfolding Protein (iCAMP) Assay with High Sensitivity for Assessing the Completion of BSE Prion Destruction

Abnormal isoform of prion proteins (*e.g.*, PrP^{sc}) retain infectivity even after undergoing routine sterilization processes. A sensitive method to detect the infectivity is a bioassay. However, the result of such bioassay can only be obtained after several hundred days. Hence, cyclic amplification of misfolding protein (CAMP) provides an attractive alternative in which PrP^{sc} can be amplified *in vitro* for assessing prion inactivation. Since three rounds of CAMP require only about 6 days, CAMP is much faster than the traditional bioassay.

An *in vitro* cyclic amplification mis-folding protein (iCAMP) method is developed herein for assessing the completion of BSE prion decontamination in TAD. Briefly, a 10% (w/v) homogenate of normal bovine brain and bovine brain with BSE is prepared in a conversion buffer. Specifically, iCAMP is set up with a volume of 50 µL containing different amounts of BSE prion (0.0001 to 1 g of the tissue equivalent) and a comparable amount of 10% (w/v) normal brain homogenate substrate. Amplification is conducted using a programmable sonicator with microplate horn (*e.g.*, a Misonix S-3000 model) at 37°C. Amplification parameters are optimized using the following conditions: cycles: 40 to 150; power-on: 90 to 240 W; pulse-on time: 5 to 20 seconds; and interval: 30 to 60 minutes. Results of iCAMP are confirmed with WB (Western Blot) and PK digestion.

In the assessment strategy, if no BSE prion is detectable in TAD post-digest samples by WB, the sample is subjected to amplification using iCAMP. Purified post-digest samples is used as the "seed," with 10% (w/v) bovine brain homogenate containing PrP^c as the substrate for iCAMP amplification. A serial dilution of brain homogenate containing BSE serves as a positive control. If a single motif of a mis-folded BSE prion protein still exists, the quantity of misfolding BSE prion is exponentially augmented by iCAMP. The sensitivity of iCAMP enables detection of a single motif of BSE prion protein (see Mahayana *et al.*, *Brioche Biophysics Rees Common* 348: 758 -762, 2006). If residual BSE is not detectable after 150 cycles, it indicates that BSE has been eradicated completely by the TAD process. iCAMP enables quick and efficient screening for a potential residual of BSE prion in post-digest samples, thus saving time and money that would otherwise be spent in animal-based bioassay.

Intracerebral inoculation of prions into mice or hamsters is a typical bioassay for assessing the infectivity of PrP^{sc} (Scott *et al.*, *Arch Virol (Suppl)* 16: 113-124, 2000). Bioassay of BSE decontamination is conducted on those samples verified by iCAMP as "not detectable" using the transgenic mouse model. Transgenic (Tg) mice over-expressing full-length bovine PrP (Tg BoPrP) or inbred transgenic mouse is used for this purpose because of their susceptibility to BSE infection (Scott *et al.*, *Proc Natl Acad Sci USA* 94: 14279-14284, 1997; Scott *et al.*, *J Virol* 79: 5259-5271, 2005). Specifically, about 50 µL of filtrate-sterile iCAMP-negative sample is inoculated into mouse brain via a trephine of the skull under sterile conditions. Observation continues for 250 days or until clinical signs are developed. Some of the low-grade positive samples detected by WB, and WB

negative/iCAMP positive samples is also subjected to mouse bioassay (Figure 3, strategy of assessment). These assays enable determination of whether the infectivity of BSE prion has been eliminated or altered in TAD process post-digestively. Brain samples are taken for immunohistochemistry confirmation of disinfection of BSE using specific antibodies (Andréoletti, PrP^{sc} immunohistochemistry. In *Techniques in Prion Research*, Edited by Lehmann S and Grassi J, p 82, Birkhauser Verlag, Basel, Switzerland, 2004).

Example 4 Mechanisms of BSE Prion Disinfection in TAD

Complete decontamination of infectivity of BSE prion in TAD is expected to result from either entire degradation of or substantial structural and conformational changes to BSE prion proteins (Paramithiotis *et al* , 2003, Brown, 2003, Alexopoulos *et al*, 2007). These changes are investigated further using conformational assays and state-of-the-art mass spectrometry (Moroncini *et al*, 2006, Domon and Aebersold, 2006).

Mass spectrometry (MS) can determine peptide covalent structures and their modifications. Proteins from the post-digest samples are isolated, fractionated and digested to the peptides (Lo *et al*, 2007, Reiz *et al*, 2007a). A shotgun and/or comparative pattern analysis is used in MS analysis. Relative quantification of proteomic changes of any two comparative samples, such as digested and undigested ones, are carried out using differential stable isotope labeling of the peptides in the two samples followed by liquid chromatography MS (LC-MS) analysis (Ji *et al*, 2005a.b.c). This method is selective to detect and quantify only the proteins with abundance and/or sequence alternations in the two samples. Recent research has shown that various prion constructs including mis-folded prion aggregates can be digested sufficiently with or without trypsin, and 100% sequence coverage was obtained using the microwave-assisted acid hydrolysis (MAAH) (Zhong *et al*, 2004 and 2005; Wang *et al*, 2007; Reiz *et al*, 2007b).

To determine if BSE prion is degraded by TAD, structural alternation from amino acid modification and/or conformational change are probed by using MAAH, isotope labeling, LC-MS and/or MS/MS. If BSE prion is degraded by TAD, the resulting peptides can be identified by LC-MS/MS, which is useful in determining the potential protease(s) involved in cleaving the specific amino acid site(s).

Thermophilic anaerobic bacteria and their proteases play a significant role in destruction of BSE prions. A number of anaerobic bacterial species in the TAD digester

containing BSE prion are identified with real time-PCR based genotyping of 16S ribosomal RNA gene (Ovreås *et al*, 1997). Functional analysis of proteolytic activities within the supernatant of the TAD-BSE mixture and/or of the bacterial isolates is carried out using the azocoll assay (Chavira Jr *et al*, 1984, Myller-Hellwig *et al*, 2006). All these analyses facilitate the understanding of the mechanism(s) of BSE prion destruction, which may lead to the optimization of BSE decontamination strategy and potential drug discovery for prion-associated disorders.

Example 5 Using Protein-enriched and Decontaminated BSE Prion-Containing Materials as Feedstock to Increase the Fuel Value of Biogas

Preliminary results demonstrated the protein-load dependent-increase of biogas production (CO_2 plus CH_4) in the pilot study on scrapie inactivation (see Example 1). Accumulated methane in TAD containing high- and low-doses of scrapie and control brain tissue was about 2.75- and 1.70-folds higher respectively than that in TAD control without proteins during a course of digestion (Figure 2).

In this experiment, biogas production profiles from TAD digesters containing BSE brain alone and BSE brain tissue mixed with other types of the tissues defined as SRM are compared. If the biogas profiles do not show differences, it indicates that anaerobic microbes treat different sources of tissue-derived proteins in a similar way. The comparative results of WB provides further evidence of whether decontamination of BSE prion is compromised by mixing the BSE brain tissue with other types of SRM tissues in TAD digester. It has been suggested that increased levels of ammonia due to protein/amino acid enrichment in the digestate inhibits TAD (Sung and Liu, 2003; Hartmann *et al*, 2005). In order to mitigate this effect (if any), the amount of protein load as feedstock in TAD can be optimized using existing computerized pilot plan and in the batch digester, respectively.

To further improve the system, ammonia in the biogas can be stripped during the TAD process. For example, ammonia can be captured by any ammonia-sorption materials (such as those described in US20080047313A1, incorporated by reference), which will turn ammonia (NH_3) into $(NH_4)_2SO_4$ or other compounds. The captured ammonia (such as $(NH_4)_2SO_4$) can be integrated into TAD effluent and then further processed to produce biofertilizer. This integrated technology will not only ensure productivity of the TAD

process and high efficiency of BSE prion destruction, but will also increase biogas fuel value and market value of TAD effluents as a biofertilizer.

Example 6 Inactivation of Viruses Using Thermophilic Anaerobic Digestion

This example provides evidence that the thermophilic anaerobic digestion (TAD) process is capable of inactivating a model virus and its infectivity. The example also provides data concerning the dose- and time-dependent inactivation of TAD on the model virus. Furthermore, the example provides a platform to investigate the specific component(s) of TAD (*e.g.*, enzyme, VFA, temperature, pH.) that plays a role in viral disinfection.

The model virus used in the study is the Avian Herpesvirus (ATCC strain N-71851), a DNA virus. This virus causes outbreaks of infectious avian laryngotracheitis (ILT) and death of chicken. Susceptible cell line used in the study is LMH (ATCC CRL-2117), a hepatocellular carcinoma epithelial cell line. Infection of the LMH cell culture *in vitro* by the avian herpesvirus induces cytopathic effects (CPE, or cell death).

According to the study design, concentrated infectious viral stock was prepared by incubating ILT virus-infected LMH cell culture at 37°C and under 5% CO_2. The resulting concentrated infectious viral stock was mixed with TAD filtrate, which was obtained by centrifuging a TAD digestate (55°C anaerobic digestion), and filtering the supernatant through a 0.45 μm and a 0.22 μm filter, respectively. The mixture was allowed to be incubated at 37°C for varied times (see below).

After incubation, a fixed amount of an aliquot of the mixture was applied to a monolayer of LMH cells grown on cover slips. The cells were then incubated at 37°C for about 24 - 72 hrs, and the results examined under the microscope.

The results showed that a mere 30-minute pre-incubation of the ILTV stock with the TAD (thermophillic anaerobic digestion) sludge (centrifuged at about 10,000 x g and filtered through 0.45 and 0.22 μm filters, either with or without neutralizing pH (original pH ~ 8.0)) aborted the appearance of CPE in the cultured LMH cells. This result indicates that some molecules in the filtrate of the TAD inhibited or inactivated ILTV, since the titrate was devoid of any live bacteria or virus after the double filtration.

The dose-dependent viral inactivation by TAD filtrate after 30-min. pre-incubation was also measured. The results show that the tissue culture infection dose ($TCID_{50}$) for ILTV was 10^8 dilution of stock virus. Wide-spread CPE occurred at 2 days at 1:1 ratio of ILTV stock : TAD filtrate. Moderate CPE occurred at 4 days at 1:4 ratio of ILTV stock : TAD filtrate. In contrast, no CPE occurred at 1:10, 1:20, or 1:100 ratio of ILTV stock : TAD filtrate. The results were summarized in the table below.

Table 2. Dose-dependent viral inactivation

Dose	Day 1 (PS infect)	Day 2 (PS infect)	Day 3 (PS infect)	Day 4 (PS infect)
1 part virus/1 part TADF	V-	V+; CPE 25%	V+; CPE 50%	V+; CPE 75%
1 part virus/2 parts TADF	V-	V+; CPE 25%	V+; CPE 50%	V+; CPE 75%
1 part virus/5 parts TADF	V-	V-	V-	V+; CPE 25%
1 part virus/10 parts TADF	V-	V-	V-	V-; No CPE
1 part virus/100 parts TADF	V-	V-	V-	V-; No CPE
1 part virus/1 part PBS	V+	V+; CPE 25%	V+; CPE 50%	V+;CPE>90%
1 part PBS/1 part TADF	V-	with good cell monolayer (no viral ctrl)		

* Detectable $TCID_{50}$ was 1×10^{-8}

Time-dependent viral inactivation by TAD filtrate : ILTV stock at 1:1 ratio were also investigated. It was found that wide-spread CPE occurred in inoculated culture at 2 days after incubation of viral stock with TADF for 0, 10, 30 minutes at 37°C. Moderate CPE occurred in inoculated culture at 3 days after incubation of viral stock with TADF for 60 minutes at 37°C. Minimal CPE occurred in inoculated culture at 3 days after incubation of viral stock with TADF for 120 minutes at 37°C. The results were summarized in the table below.

Table 3. Time-dependent viral inactivation

Time	Day 1 (PS infect)	Day 2 (PS infect)	Day 3 (PS infect)	Day 4 (PS infect)

0 min.	V-; CPE -	V+; CPE 25%	V+; CPE 50%	V+; CPE 75%
10 min.	V-; CPE -	V+; CPE 25%	V+; CPE 50%	V+; CPE 75%
20 min.	V-; CPE -	V?; CPE<25%	V+; CPE 25%	V+; CPE 75%
60 min.	V-; CPE -	V-; CPE -	V+; CPE 25%	V+; CPE 50%
120 min.	V-; CPE -	V-; CPE -	V+; CPE < 25%	V+; CPE 25%
120 min. (PBS + virus)	V-; CPE -	V+; CPE 25%	V+; CPE 50%	V+; CPE 75%

* ILTV: AD filtrate = 1:1

Results in Tables 2 and 3 are summarized in Figure 4.

The experiments described in this example provide evidence that TAD filtrate alone (without anaerobic bacteria) can eliminate the infectivity of ILT virus in a dose-and time-dependent manner, when the infectious viral stock was pre-incubated with the filtrate. Although proteases or other bioactive enzymes in TAD filtrate do not seem to be major attributing factors to viral inactivation, volatile fatty acid (VFA) at given concentration (*e.g.*, > 250 ppm) might play a role in viral inactivation.

Although the experiments used ILT virus, other viruses, especially other DNA viruses in the same family (including human viruses) can also be effectively destroyed in TAD process described herein. While not wishing to be bound by any particular theory, viral destruction may be a result of a synergistic effect between small metabolic molecules and complex anaerobic bacterial colonies in the TAD digestion system.

The exact identity of the small molecules critical for viral disinfection may be determined using any art-recognized methods, such as GS-MASS or HPLC-MASS, and nucleic acid testing.

Example 7 Removal of Infectivity of Infectious Laryngotracheitis Virus (ILTV) Using Thermophilic Anaerobic Digestion (TAD) Process

Infectious laryngotracheitis (ILT) is an upper-respiratory disease of poultry caused by a herpesvirus. It is a provincially reportable disease in Alberta, Canada. Because of its endemic nature, it is economically important to the provincial poultry industry. In areas of intense poultry production and during disease outbreaks, the virus causes significant loss of the birds and reduction in egg production.

The virus can survive in tracheal tissues of a bird up to 44 hours post mortem. Although ILT virus (ILTV) can be inactivated by organic solvents and high temperature (55°C and above), the TAD process described herein provides a more cost-effective and environmentally responsible way to destroy this virus.

In this experiment, ILTV was successfully cultured in specific pathogen-free chicken embryos and an avian continuous cell line (chicken lung cell). The cells are highly susceptible to the virus, and exhibit characteristic cytopathic effects (CPE) 3 to 4 days post infection. The ILTV infected cells can readily be identified directly under microscope or using an indirect fluorescent test (IFAT).

In the first set of experiments, an equal volume of ILTV (challenge dose of 100,000 TCID 50) and the filtrate from active TAD (TAD-f) digestate (collected from the Integrated Manure Utilization System (IMUS^{TM}) demonstration plant, Vegreville) (TAD-f) were mixed and incubated at 37°C for different periods of time (10, 30, 60 and 120 min.) before inoculation into the tissue culture cells. In the second set of experiments, TAD-f was mixed with 1 volume of virus suspension at different ratio of digestate vs. virus (1:1, 25:1, and 100:1) and incubated for 60 minutes before inoculation into the tissue culture cells. The control used for comparison was an untreated virus suspension with identical infectious dose inoculated into the cell line. The CPE of the cell cultures were scored after 3 to 4 days. The different incubation times and concentrations of TAD-f used were converted into log 10 and plotted against the percentages of CPE observed (data not shown).

We observed that, after an incubation period of 2 hours (120 min.), and similarly using the ratio of 100 times of TAD-f to 1 volume of virus suspension, the ILTV CPE has been eliminated, indicating that the infectivity of ILTV was removed completely. The percentages of CPE of ILTV were inversely proportional to the incubation time and amount of TAD-f added.

We have successfully demonstrated here a simple, inexpensive, and environmentally friendly TAD technology for disinfection of ILTV. In addition, the thermophilic anaerobic digestion system has been proven to generate renewable energy via biogas and reduce green-house gas emissions and the foot-print of agri-biowaste in the feedlot practice. Viral removal by TAD provides another environmentally friendly

alternative to the poultry industry for controlling spread of ILT, and management of agri-biowaste.

Example 8 Evaluation of Pathogen in Biowaste and Digestate

There are many different types of waste products that are used for anaerobic digestion, however, biowaste that contains manure has a high density of coliform bacteria (1-6). The coliform bacteria can include pathogens associated with human illness, such as *Salmonella* and other zoonotic pathogens such as *Campylobacter* and *Listeria* (7-10). Generally, methods used to denote contamination in waste use indicator organisms like fecal coliform bacteria. For water, detection and enumeration of this group of organisms are used to determine the suitability of water for domestic and industrial use (11). In the United States, sludge from wastewater treatment plants must fulfill the density requirements from the US Environmental Protection Agency (USEPA) for fecal coliform as an indicator or *Salmonella* as a pathogen (12).

In the discussion presented by Pell (13) on pathogenic microbes in manure, there is mention that in the past, most environmental concerns about biowaste management have focused on nutrient overload, water quality or odor problems. There are no regulations concerning pathogens in biowaste that are used for anaerobic digestion. With an emerging biogas industry in Alberta, large amounts of effluent from anaerobic digesters will be produced. There is a lack of information as to whether pathogens are present in anaerobic digester effluent and if present, whether they will pose a threat to public, animal and plant health. We have found no information on regulations for handling effluent from anaerobic digesters for Alberta, although there is information on wastewater systems (14). Alberta Agriculture and Rural Development guidelines mention that land application of digestate is under the Agricultural Operations Practices Act and Regulations as it applies to manure (15). The Canadian Council for the Ministers of the Environment (CCME), in their guidelines for organism content in compost containing only yard waste, mention that fecal coliform of fecal origin should be < 1000 Most Probable Number (MPN) /g of Total Solids (TS) calculated on a dry weight basis and *Salmonella* < 3 MPN/4 g TS (16) and compost containing other feedstock should contain fecal coliform at < 1000 MPN/g TS or *Salmonella*, < 3 MPN/4g TS. The compost with other feedstock must be exposed to 55°C or higher for a specified time depending on the type of compost.

The USEPA have imposed regulations under Title 40 of the Code of Federal Regulations (CFR), Part 503 to control the use and disposal of biosolids (17). Biosolids are defined as the recyclable organic solid product produced during wastewater treatment processes. Part 503 of the rule gives the requirements for the use of biosolids in order to prevent contamination to the public and the environment. One requirement is for the control of pathogens or disease-causing organisms and the reduction of vector attraction to the biosolids. Pathogens can be bacteria, viruses and parasites and vectors include rodents, flies, mosquitoes and disease-carrying and transferring organisms. The rules described in Part 503 ensure that pathogen levels are safe for the biosolids to be land applied or surface disposed. The criteria for biosolid Class A are the same as the CCME guidelines for compost with other feedstock, with fecal coliform < 1000 MPN/ g TS or *Salmonella* < 3 MPN/4 g TS. A biosolid is considered Class B if pathogens are reduced to levels that do not pose a risk to the public and environment. Measures must be taken to prevent crop harvesting, animal grazing and public assess to areas where Class B biosolid have been applied until the area is considered safe. The Class B biosolid requirements are that fecal coliform must be $< 2 \times 10^6$ MPN/g TS. For this biosolid, the fecal coliform is used as an indicator of average density of bacterial and viral pathogens.

We conducted a small-scale study on undigested biowaste and effluent after anaerobic digestion of biowaste using the USEPA microbiology testing methods for fecal coliform (18) and *Salmonella* (19) for biosolids and used the results to assess local biowaste samples. Due to time and resource limitations at the time of experiment, only selected analyses were performed on chosen biowaste samples.

Objectives

- to assess the levels of fecal coliform used as a contamination indicator and *Salmonella* used as pathogen indicator for selected biowaste samples
- to evaluate reduction of fecal coliform and *Salmonella* using thermophilic anaerobic digestion processes

The results from this study provide preliminary data for development of guidelines for handling and utilizing biowaste.

Biowaste and Sample Collection

All samples were collected into sterile plastic bags or bottles and tested within 2-3 hours after collection, unless otherwise stated. All samples were collected specifically for

this study except sample 1.4, which was collected and stored at ARC, Vegreville, Alberta. This sample was being used in the ARC fully automated anaerobic digestion system ARC Pilot Plant (referred to as ARC Pilot Plant from here on) at the time of this study. The digestion system operated at 55°C. All dairy and chicken manure samples were collected from the same farm in the winter months. The farm was chosen because of its close proximity to the testing laboratory, allowing valid testing of fecal coliform and *Salmonella* within the required time frame for the USEPA microbiological testing methods.

The following samples were tested in this study:

- 1.1 Dairy manure taken from within dairy cows. Three dairy manure samples collected on two occasions from 5 dairy cows. Sample 1 was a manure mixture from cows 1 and 2, and Sample 2 was a mixture from cows 3 and 4. Sample 3 was from cow 5. One sample was tested for *Salmonella* only.
- 1.2 Dairy manure from one cow that was collected from the barn and tested for *Salmonella* only.
- 1.3 Dairy manure collected from the general barn area. Some of the freshly collected manure was taken to the Edmonton ARC laboratory. The remainder of the manure was transported to Vegreville and digested in the ARC Pilot Plant. At this time the digester was running dairy manure at 55°C. The freshly collected dairy manure was fed into the digester over 10 days. The last feeding of manure was 15 hours before the sample was taken for analysis.
- 1.4 Dairy manure that was used routinely for TAD digestion at the ARC Pilot Plant. The dairy manure was collected from the same farm as samples 1.1 to 1.3 and stored for 2 months at 4°C. The stored sample and a random sample from the digester hopper were tested. The dairy manure from the hopper was diluted in the laboratory and left at 22°C for 1 hour. A post-digested sample from the dairy manure was collected and tested.
- 1.5 Chicken manure, collected from chicken cages in the barn.
- 1.6 Chicken manure, collected from the general barn area and included straw bedding.

- 1.7 Household kitchen waste, mostly vegetable and fruit waste collected daily over a 7-day period and held at 4-6°C until testing.
- 1.8 Broken eggs, including shell, collected at a grocery retail store that was close to the testing laboratory.
- 1.9 Wet distillers grain from an ethanol production plant, collected in barrels and stored at -20°C until testing in the ARC Pilot Plant. This sample was collected for use in the ARC Pilot Plant and was chosen for pathogen analysis because it was a non-manure based biowaste. A diluted sample with 8% TS was taken for fecal coliform and *Salmonella* testing.

Testing Methods

All dehydrated culture media were purchased from Neogen (MI, USA) and testing was carried out in a Biolevel II lab. A 5-tube MPN method was used as described in the USEPA methods to derive population estimates for the fecal coliform and *Salmonella*.

Total solid measurements of biowaste

Total solid analysis was done for biowaste using a forced-air oven-drying method at 70°C for 48 hours. The method assumes only water is removed. The results are reported as a percent of the sample's wet weight.

Testing for fecal coliform

The biowaste and anaerobic digester effluent were evaluated for fecal coliform using the USEPA Method 1680 (17). Briefly, the method uses a MPN procedure to derive a population estimate for fecal coliform bacteria, Lauryl-Tryptose broth and EC culture specific media and elevated temperature to isolate and enumerate fecal coliform organisms. The basis for the test is that fecal coliform bacteria, including *Escherichia coli* (*E. coli*), are commonly found in the feces of humans and other warm-blooded animals.

These bacteria indicate the potential presence of other bacterial and viral pathogens. Total solids determination was done on the biowaste samples and used to calculate and report

fecal coliform as MPN/g dry weight.

Testing for Salmonella sp.

The biowaste and anaerobic digester effluent were evaluated for *Salmonella* using the USEPA Method 1682 (18). Briefly, the method is for the detection and enumeration of *Salmonella* by enrichment with tryptic soy broth and selection with modified semisolid Rappaport-Vassiliadis medium. Presumptive identification was done using xylose-lysine desoxycholate agar and confirmation was done using lysine-iron agar, triple sugar iron agar and urea broth. Serological testing was done. Total solids were determined on a representative biowaste sample and used to calculate *Salmonella* density as MPN per 4 g dry weight.

Quality control

Milorganite (CAS 8049-99-8, Milwaukee Metropolitan Sewerage District, UNGRO Corp. ON), a heat-dried Class A biosolid proven by USEPA was used and spiked with appropriate control bacteria. *E. coli* (ATCC# 25922) was used as the positive control for the fecal coliform test and negative control for the *Salmonella* test. *Salmonella typhimurium* (ATCC# 14028) was used as the positive control for the *Salmonella* test.

Enterobacter aerogenes (ATCC# 13048) and *Pseudomonas* (ATCC# 27853) were used as negative controls for the fecal coliform test.

Results and Discussion

The table below gives the total solid, fecal coliform and *Salmonella* MPN for the biowaste samples.

Summary of microbiology testing results of selected biowaste samples

	Samples	Total solids (% of wet weight)	Fecal coliform (MPN/g TS)	*Salmonella* (MPN/4g TS)
1.1	Dairy manure taken from within dairy cows			
	Sample 1	13	5.6×10^6	< 0.18
	Sample 2	15	1.1×10^7	< 0.18
	Sample 3	14[a]	Not done	< 0.18
1.2	Dairy manure from general barn area			
		14[a]	Not done	< 0.18
1.3	Dairy manure from general barn area			
		15	1.1×10^7	4.0×10^0

	Anaerobic digestion effluent of dairy manure after 15 hrs digestion			
		10	< 0.18	< 0.18
1.4	Dairy manure used at ARC Pilot Plant			
	Dairy manure stored for 2 months at 4°C			
		14	8.8×10^4	< 0.18
	Dairy collected from ARC Pilot Plant hopper before anaerobic digestion			
		10	1.8×10^4	2.1×10^0
	Anaerobic digestion effluent of dairy manure after 15 hours hydraulic retention time			
		9	<0.18	< 0.18
1.5	Chicken manure from cages			
		37	4.3×10^6	< 0.18
1.6	Chicken manure from general barn area with straw bedding			
		78	2.1×10^6	< 0.18
1.7	Household kitchen waste			
		Not done	No growth	No growth
1.8	Broken eggs			
		Not done	No growth	No growth
1.9	Wet distillers grains			
		8	<0.18	< 0.18

a. Estimated TS values

Dairy manure samples from the same facility were tested in this study. The samples were from the general barn area and taken from within cows. When tested, the density of fecal coliform that was found in all samples ranged from 8.8×10^4 MPN/g TS to 1.1×10^7 MPN/g TS. *Salmonella*, 4×10^0 MPN/4g TS, was found in one sample collected from the general barn area. Storage of the dairy manure at 4°C for 2 months decreased the fecal coliform 2- to 3-log. In both cases where dairy manure was digested at 55°C by TAD digested for 15 hours, the fecal coliform and *Salmonella* were decreased to below detection (<0.18 MPN/g TS for fecal coliform and <0.18 MPN/4g TS for *Salmonella*).

The chicken manures, kitchen waste, eggs and wet distillers grain were not put through digestion. Both chicken manure samples had fecal coliform, 4.3×10^6 and $2.1 \times$

10^6 MPN/g TS. No *Salmonella* was detected. There were no fecal coliform and *Salmonella* in the kitchen waste, eggs and wet distillers grains.

This brief study showed that bacteria common to manures were detected in the dairy and chicken manure samples. According to the USEPA guidelines for a Class A biosolid, the fecal coliform density was above the accepted level in all manure samples, and for a Class B biosolid, the fecal coliform density was above the accepted level in the freshly collected manure samples. The increased fecal coliform levels indicate that pathogenic bacteria could be present in these samples. This was verified by the fact that one fresh dairy sample contained 4.0×10^0 MPN/4g TS and a random hopper sample from the ARC Pilot Plant contained 2.1×10^0 MPN/4g TS *Salmonella*. The sample was tested to contain below detection levels of both fecal coliform and *Salmonella* after anaerobic digestion at 55°C for 15 hours.

Bendixen (20) looked at the animal and human pathogen reduction in Danish biogas plants. It was reported that pathogen survival was greatly reduced at thermophilic digestion temperatures (50°C to 55°C) but not at low and mesophilic temperatures (5°C to 45°C). Biogas plant construction, function and management need to be monitored in order to assure pathogen destruction and policies need to be in place to classify the digested effluent for proper disposal. The requirements in the USEPA standards (17) for sewage sludge use and disposal indicate that sewage sludge should be analyzed for enteric viruses and viable helminth ova. There are also requirements given for vector attraction reduction and reduction of volatile solids. As well, other pathogens should be investigated. For example, human norovirus strains have been found in livestock, indicating a route for zoonotic transmission (21). As well, policies have been made concerning plant pathogens that relate to anaerobic digestion facilities in Germany (22).

Summary

- Using the USEPA Class A biosolids and CCME guideline for compost of <1000 MPN/g TS for fecal coliform, all the freshly collected manures (dairy and chicken) were above the accepted level.

- Using the USEPA Class B biosolids guidelines of $<2 \times 10^6$ MPN/g TS for fecal coliform, all the freshly collected manure samples (dairy and chicken) were above the accepted level.

• For one fresh dairy manure, the *Salmonella* exceeded the USEPA Class A biosolids and CCME guideline for compost of <3 MPN/4 g TS.

• Storage of dairy manure at 4°C for 2 months decreased fecal coliform concentration.

• Anaerobic digestion at 55°C for 15 hours reduced fecal coliform and *Salmonella* to below detection levels. Fifteen hours of digestion in a continuous stirred tank reactor system appeared to be adequate for reduction.

• Household kitchen waste, broken eggs and wet distillers grains contained either no fecal coliform and *Salmonella* or levels below detection using the MPN method.

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Example 9 Enhanced Prion Destruction Using Thermophilic Anaerobic Digestion (TAD) Process

Applicants demonstrate in this example that prion destruction is also enhanced by adding carbohydrate-based substrate (non-protein substrate) into the digester and keep a consortium of anaerobes in active status.

Applicants previously showed that, biogas profile (CH_4 and CO_2) in batch digestion reached a peak at day 8 to 11, and then quickly dropped to a baseline level without further addition of substrate into the digestion. This result indicates that most of the anaerobes were in the resting state after the leveling off occurred.

In this study, cellulose substrate was added periodically (about every 7 days) starting day 11 into one study group of TAD digestion with 10 ml of 40% scrapie brain tissue. As a control, another study group was similarly set up (TAD digestion with 10 ml of 40% scrapie brain tissue), but without the additional of additional cellulose substrates, as in the previous study. The study was carried on for 90 days. Sampling schedule was as follows: day 0, 6, 11, 18, 26, 40, 60 and 90. At the end of the study, the scrapie prion was extracted, purified, desalted, and concentrated for analysis using 12% SDS-PAGE and

Western blot. Western blot images were semi-quantified using Alpha Innotech Image Analyzer (MultiImage II, Alpha Innotech, San Leandro, CA).

The results from the image analysis show the following:

1) In the control group of TAD with scrapie prion only (no added cellulose substrate), 2.2 log reduction of scrapie prion was achieved at day 26 comparing to the starting amount of scrapie prion in TAD at day 0, and the amount of scrapie prion spiked in phosphate buffer (PBS) at day 26, respectively. This result was the same as shown in the previous study.

2) In the group of TAD with scrapie prion and additional cellulose substrate, more than 3 logs of reduction of scrapie prion was achieved at day 26 comparing to the starting amount of scrapie prion in TAD at day 0, and the amount of scrapie spiked in PBS at day 26, respectively.

3) TAD only eliminated 0.8 logs of scrapie prion (from 12.18 to 11.38 logs of integrated density and area (IDA)) while and TAD with additional cellulose substrate (1 gram in 60 ml of TAD/scrapie prion mix) eliminated 1.37 logs of scrapie prion (from 12.15 to 10.78 logs of IDA) ($p < 0.001$, student-t test), from day 11 to 18.

4) TAD eliminated 1.05 logs of scrapie prion (from 11.38 to 10.34 logs of IDA), while TAD with the second cycle of additional cellulose substrate eliminated scrapie prion to undetectable level in the current Western blot method, from day to 18 to 26. It is expected that more than 2 log further reduction could be achieved during this period after the second addition of cellulose substrate (Figure 1. Western blot image showing the reduction of scrapie prion from day 11 to day 26).

5) A computational modeling is being carried out to predict destruction rate of scrapie prion using TAD process with and without addition of carbohydrate-based substrate. The modeling allows Applicants to avoid the limitation of detection sensitivity using the current available methods in the field of prion disease research and diagnostics.

In summary, the subject TAD technology can effectively destroy scrapie prion proteins in a time-dependent manner. Adding carbohydrate-based and non-protein containing substrates periodically into TAD process enhanced destruction capability. It is estimated that more than 3 logs of reduction of scrapie prion titers was obtained at day 26 in the group with additional carbohydrate-based (non-protein containing) substrates. Based on the experimental data, a computational modeling can be used to predict the time

course of prion reduction in TAD process, and the time it takes to achieve substantially complete eradication of prion in SRM.

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CLAIMS:

1. A method for reducing the titer of a biohazard that may be present in a carrier material, comprising providing the carrier material to an anaerobic digestion (AD) reactor and maintaining the rate of biogas production substantially steady during the AD process.

2. The method of claim 1, wherein the biohazard comprises hormones, antibodies, body fluids, viral pathogens, bacterial pathogens, and/or weed seeds.

3. The method of claim 1, wherein the bio-hazard comprises prion.

4. The method of claim 3, wherein the prion is scrapie prion, CWD prion, or BSE prion.

5. The method of claim 3 or 4, wherein the prion is resistant to proteinase K (PK) digestion.

6. The method of any of claims 1-5, wherein the carrier material comprises a protein-rich material.

7. The method of any of claims 1-6, wherein the carrier material comprises a specified risk material (SRM).

8. The method of claim 7, wherein the SRM comprises CNS tissue (*e.g.*, brain, spinal cord, or fractions / homogenates / parts thereof).

9. The method of any of claims 1-8, wherein the AD reactor is operated in batch

mode, semi-continuous mode, or continuous mode.

10. The method of claim 9, wherein the batch mode lasts less than about 0.5 hr, 1 hr, 2 hr, 5 hr, 10 hr, 24 hr, 2, 3, 4, 5, 6, 7, 10, 20, 30, 40, 50, or 60 days.

11. The method of claim 9 or 10, wherein the rate of biogas production peaks at about 0.5-5 hrs, 1-7 days, or 5-10 days after the beginning of the batch mode operation.

12. The method of any of claims 1-11, wherein a carbon-rich material is provided, semi-continuously to the AD reactor once every about 0.5-5 hrs, 1-7 days, or 5-10 days after reaching peak biogas production, to maintain substantially steady biogas production.

13. The method of claim 12, wherein the carbon-rich material comprises fresh plant residues or other easily digestible cellulose.

14. The method of any of claims 1-13, wherein the AD reactor contains an active inoculum of microorganisms at the beginning of the batch mode operation.

15. The method of any of claims 1-14, wherein the AD process is carried out by a consortium of anaerobic microorganisms, such as psyclophilic microorganisms, mesophilic microorganisms, or thermophilic microorganisms.

16. The method of claim 15, wherein the thermophilic microorganisms are acclimatized with substrates containing proteins with abundant β-sheets.

17. The method of claim 15, wherein the thermophilic microorganisms are acclimatized by culturing with substrates containing amyloid substance at elevated

temperature and extreme alkaline pH.

18. The method of any of claims 1-17, further comprising adding one or more supplemental nutrients selected from Ca, Fe, Ni, or Co.

19. The method of any of claims 1-18, wherein the AD is carried out at about 20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C, or above.

20. The method of any of claims 1-19, wherein 2 logs or more reduction of the titer of the biohazard is achieved after about 30 days or 18 days of anaerobic digestion.

21. The method of any of claims 1-20, wherein 4 logs or more reduction of the titer of the biohazard is achieved after about 30 or 60 days of anaerobic digestion.

22. A method for producing biogas, comprising providing to an anaerobic digestion (AD) reactor a protein-rich feedstock, wherein the rate of biogas production is maintained substantially steady during the AD process.

23. The method of claim 22, wherein the AD reactor is operated in batch mode.

24. The method of claim 23, wherein the AD reactor contains an active inoculum of microorganisms at the beginning of the batch mode operation.

25. The method of claim 23 or 24, wherein the batch mode lasts less than about 0.5 hr, 1 hr, 2 hr, 5 hr, 10 hr, 24 hr, 2, 3, 4, 5, 6, 7, 10, 20, 30, 40, 50, or 60 days.

26. The method of any of claims 22-25, wherein the rate of biogas production peaks at about 0.5-5 hrs, 1-7 days, or 5-10 days after the beginning of the batch mode operation.

27. The method of any of claims 22-26, wherein a carbon-rich material is provided, semi-continuously to the AD reactor once every about 0.5-5 hrs, 1-7 days, or 5-10 days after reaching peak biogas production, to maintain substantially steady biogas production.

28. The method of claim 27, wherein the carbon-rich material comprises fresh plant residues, or other easily digestible cellulose.

29. The method of any of claims 22-28, wherein the protein-rich feedstock comprises hormones, antibodies, viral pathogens, or bacterial pathogens.

30. The method of any of claims 22-29, wherein the protein-rich feedstock is a specified risk material (SRM).

31. The method of claim 30, wherein the SRM comprises one or more prions or pathogens.

32. The method of claim 31, wherein the prions comprise scrapie, CWD, and/or BSE prion.

33. The method of claim 31, wherein the prions are resistant to proteinase K (PK) digestion.

34. The method of any of claims 30-33, wherein the SRM comprises CNS tissue (*e.g.*, brain, spinal cord, or fractions / homogenates / parts thereof).

35. The method of any of claims 31-34, wherein 2 logs or more reduction of the titer of the prions is achieved after about 30 days or 18 days of anaerobic digestion.

36. The method of any of claims 31-35, wherein 3 logs or more reduction of the titer of the prions is achieved after about 30 or 40 days of anaerobic digestion.

37. The method of any of claims 31-36, wherein 4 logs or more reduction of the titer of the prions is achieved after about 30 or 60 days of anaerobic digestion.

38. The method of any of claims 22-37, wherein the AD is carried out at about 20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C, or above.

39. The method of any of claims 22-38, wherein the bacteria carrying out the AD comprise a consortium of thermophilic microorganisms, and/or a consortium of anaerobic microorganisms, such as psyclophilic microorganisms, mesophilic microorganisms, or thermophilic microorganisms.

40. The method of any of claims 22-39, wherein the bacteria carrying out the AD is acclimatized with substrates containing proteins with abundant β-sheets.

41. The method of any of claims 22-40, wherein the bacteria carrying out the AD is acclimatized by culturing with substrates containing amyloid substance at elevated temperature and extreme alkaline pH for 3 months.

42. The method of any of claims 22-41, further comprising adding one or more supplemental nutrients selected from Ca, Fe, Ni, or Co.

43. A method for reducing the titer of a viral biohazard that may be present in a carrier material, comprising contacting the carrier material to a liquid portion of an anaerobic digestion (AD) digestate, preferably a thermophilic anaerobic digestion (TAD) digestate.

44. The method of claim 43, wherein the contacting step is carried out at 37°C or room temperature (*e.g.*, about 20-25°C).