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ORIGINAL PAPER

Modeling effects of granules on the start-up of anaerobic digestion of dairy wastewater with Langmuir and extended Freundlich equations

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Abstract The effects of granules-inocula on the start-up of anaerobic reactors treating dairy manure were studied in a batch-fed reactor. The effects of start-up period and ratio of granules to feed were analyzed. Results indicated that the effects of start-up period could be described by Langmuir model, while the Extended Freundlich model could be used to model the effects of ratio of granules to feed on cumulative biogas production. In addition, transmission electron microscopes (TEM) and scanning electron microscope analysis were conducted to elucidate the distribution of microbial population and micro-colonies in granules and manure. From the TEM micrographs analyses, the ratios the *Syntrophobacter* and methanogens in granule and manure were shown to be 1.57 ± 0.42 and 0.22 ± 0.20, respectively. These results demonstrated that granules-inocula could reduce the period required for onset of biogas by 25%.

Keywords Start-up · Anaerobic digestion · Granules · Dairy manure · Langmuir model · Extended Freundlich model · SEM and TEM

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Introduction

Dairy cattle in the US generate an estimated 2×10^9 tones of solid waste (manure) annually [1] much of which could be utilized for methane production by converting it anaerobically into biogas. Use of anaerobic digestion can contribute to the green economy, a future agenda of US. Improvements to the anaerobic process in terms of reducing the start-up period, improving biogas production rate, and preventing reactor failure are important issues in order to promote widespread adoption of this technology. This research addresses both the start-up period and the rate of biogas production rate.

The start-up period is the initial period after commissioning of the digester and is generally considered to be the most critical step in the operation and maintenance of anaerobic digesters. The fate of anaerobic digester in terms of treatment efficiency and its economics is determined during the start-up period [2, 3]. During this period acclimatization of biomass in a new environment takes place and must be done properly to enhance start-up. Incomplete biomass acclimatization can cause delayed biomass growth, which often results not only in poor reactor performance but also probably in the complete failure of the reactor.

Sewage sludge and dairy manure are often used as an inoculum to start-up anaerobic reactors [2–5]. Granules, with self-immobilized and well-structured microbial population formed in upflow anaerobic sludge batch reactor (UASB), is another possible inoculum. However, the use of granules as an inoculum in dairy manure digestion has not been reported. The advantages of granules compared to other inocula are: higher biomass concentrations and their well structured microbial distribution [6, 7]. Granules maintain their microbial structure even under high shear stress conditions [3, 8], which enhances anaerobic process.

Approaches such as physical (selection pressure theory), microbial (Cape Town Hypothesis, spaghetti theory), and thermodynamic (crystallized nuclei formation theory) have been proposed to describe anaerobic sludge granulation [9]. Under selection pressure, continuous selection of sludge particles is considered as the essence of the granulation. Cape Town Hypothesis proposed that granulation depends on Methanobacterium strain that utilizes H_2 as energy sources to produce amino acids. Spaghetti Theories proposed that granulation is two steps process: (1) precursors formation (aggregation of methanothrix), and (2) actual growth of granules from precursor (growth of individual bacteria and entrapment of non-attached bacteria). Thermodynamics theories explain granulation by considering hydrophobicity and electrophoretic mobility. Microbial theories explain sludge granulation based on the granule structure and characteristics of certain microorganisms.

Electron micrographs such as transmission electron microscopes (TEM) and scanning electron microscope (SEM) have been widely used for the identification of different microbial populations in granules [10]. The micrographs provide a clear description of granule structure and microbial ecology. TEM and SEM micrographs have shown that granules consist of a vast diversity of microorganisms with Methanosaeta-like cells dominating in the granules core [10]. TEM micrographs of anaerobic granules formed in granular bed baffled reactors showed three microbial zones. While facultative bacteria are found in the outer layer, Methanosaeta bacteria were dominant in the core zone [11]. The role of substrate degradation and diffusion kinetics on granule structure was well explained using SEM and TEM micrographs [12]. A composition and characteristics analysis of granular sludge through SEM and TEM micrographs also showed that organic loading rate and feed type influence the methanogens population in granules [13].

The applications of Langmuir and Freundlich equations have been reported in various fields. In biosorption system, for example, Langmuir and Freundlich equations have been found most suitable for equilibrium analyses [14]. The application of Extended Freundlich model in various studies such as phosphate sorption by soil [15], biosorption of heavy metals from waste water using *Pseudomonas* spp. [16], and removal of cadmium from water environment by adsorption on biofilter [17] has been reported. It has also been used in studying uranium and vanadium sorption by chitosan and derivatives [18]. Kinetics of endoglucanase and endoxylanase uptake by soybean seeds [19] and cadmium removal from water by biofilm covered granular activated carbon [20] are the other areas where extended Freundlich model has been reported. Adsorption models (Langmuir and Freundlich) provide an adequate description of the equilibrium between absorbed metal ions on the solid surface and metal ions in solution at a constant temperature. Traditionally, these isotherms have been used to represent the amount of metal removed as a function of the equilibrium concentration of metal ions in solution for optimizing the process. This study explored the application of these isotherms to represent the release of methane from dairy manure in anaerobic digestion process (at constant temperature) under different inoculum concentrations.

The goal of this research was to reduce the start-up period and improve the biogas production rate. In addition, microbial distributions in manure and granules were determined using SEM and TEM in order to understand the interaction between microbial populations. Cell counting based on the TEM image analysis was used to estimate the ratio between methanogens and *Syntrophobacter* in granule and manure samples. Finally, equilibrium models (Langmuir, Freundlich, and extended Freundlich models) were developed to model the start-up of AD process with granules-inocula.

Material and method

Dairy manure (feedstock) and inoculum characteristics

Raw feedstock was collected from the dairy center at Washington State University in Pullman, WA. It was stored at −20 °C. Just before the treatments preparation; feedstock was transferred from −20 to 4 °C in order to thaw it. The feedstock was then sieved through a mesh of 850 μm (USA standard test sieve, No 20, S/N 04106855, Fisher Scientific Company) to remove large debris and fiber in order to minimize blockage of the lab-scale reactors. The total solids (TS) and volatile solids (VS) of the feedstock were 0.87 (±0.17) % (g/g) and 0.54 (±0.09) % (g/g), respectively.

Granules collected from a UASB reactor treating potato starch waste (Penford Food Ingredients Co., Richland, WA) was used to inoculate the reactors. The inocula were stored at −20 °C. A day before starting the experiments granules was transferred to a 4 °C environment in order to thaw it. The TS and VS of the granules were 6.32 (±0.32) % (g/g) and 5.37 (±0.32) % (g/g), respectively.

Experimental design

Batch anaerobic digestion tests for six levels of inoculums were conducted at 35 °C in six identical batch reactors with a working volume of 125 ml (Serum bottles, Scientific Instrument Services, Ringoes, NJ). These six reactors digested the feed material for 60 days. After starting the experiment, 5% of the slurry in the reactors was taken for liquid phase analysis, which reduced the reactor feed

material with time. However, precisely the same amounts of samples were taken out from all reactors on same day to provide similar feed volume to all treatments. This provided the biogas production in volumetric unit (ml of gas/ml of feed material) for comparing the treatments. After collecting the gas samples from gas phase, the remaining gas in the reactors was released daily to ensure accurate gas production results. Six levels of inoculations were prepared by mixing inoculum (granules) and manure (feedstock) at different ratios (on volume basis). The six levels of inoculations were: (1) 0% of inoculum and 100% of feedstock (control); (2) 7% inoculum and 93% of feedstock; (3) 12% inoculum and 88% feedstock; (4) 15% inoculum and 85% of feedstock; (5) 18% of inoculum and 82% of feedstock; and (6) 20% inoculum and 80% feedstock.

After filling the prepared mixtures (80 ml) in the reactors, the reactors were sealed with a rubber septum and flushed with a gas mixture of 80% N_2 and 20% CO_2 for 5 min in order to ensure anaerobic conditions. An orbital shaker (New Brunswick Scientific, Edison, NJ) running at 125 rpm was used to stir the reactors. Slurry samples from the reactors was taken and used for TS, VS and VFA analysis. TS and VS were analyzed in accordance with standard methods [21] and the procedures for VFA analyses are described in analysis section.

The biogas produced was measured with a 35 ml gas tight glass syringe (Micro-Mate, Popper & Sons Inc., New Hyde Park, NY). Biogas samples were collected in 10 ml test vials (Labco Limited, Brow Works, High Wycombe, Buckinghamshire, UK) for biogas composition analyses. The biogas produced in reactors was released after the measurement to avoid build-up of pressure in the reactor that could lead to leakage of biogas. This procedure was applied to all the reactors.

Analysis

Methane content

Biogas composition was measured on day 2, 3, 4, 6, 8, 10, 12, 14, and 15. Biogas samples (about 7 ml) were withdrawn from the headspace of test vials and injected into a Varian CP-3800 gas chromatograph (GC) for measuring the biogas contents (methane and carbon dioxide). The GC was equipped with following detectors: Varian flame ionization detector (FID) for CH_4, Varian thermal conductivity detection (TCD) for CO_2, and a D-2 pulsed discharge helium ionization detection (HID) mode discharge detector for H_2S measurement. The GC was equipped with a switching valve (A3C6UWT) with 1 ml sample loop and a column (Varian 18′ × 18′ Hayesep Q 80/100 Mesh Silcosteel and nitrogen as carrier gas) for CO_2 and CH_4 measurement. A switching valve (A3C6UWT) with 0.25 ml sample loop and a column (Varian 50 m × 0.53 mm × 4 μm SilicaPlot and helium as carrier gas) were used for H_2S measurement. The oven and the TCD temperature were kept at 80 and 120 °C, respectively. GC was calibrated with 99.9% pure CH_4, CO_2, H_2S and H_2 standards.

Volatile fatty acids

The concentrations of volatile fatty acids (acetic, butyric and propionic acids) were determined using Dionex Ion chromatograph (DX-500 IC). The IC was equipped with AS-3500 auto-sampler. A small sample (feedstock, inoculum and digested slurry) was centrifuged at 12,000 rpm for 6 min. After centrifuging, supernatant was filtered through a 0.2-μm-pore size filter before injection into an IC. The Peak Net R software controlled the injection. A detector (ED 40 electrochemical detector) and a column (4 × 250 mm Carbon Pac^{TM} PA 10 analytical column) were used for analyzing VFA concentrations. Elution was initiated with 10% (v/v) water and 90% (v/v) 52 mM NaOH for 27.10 min, with 100 μl of sample injected for 0.10 min. The elute flow rate was 1.2 ml/min with the pressure between 1,500 and 3,000 psi. The IC was calibrated with pure acetate, butyrate and propionate standards. A verification test was performed after every 10-sample analysis.

Scanning electron microscopy

Samples were first fixed overnight at 4 °C with 2% paraformaldehyde, 2% glutaraldehyde and anaerobic 0.05 M cacodylate buffer for performing SEM analysis. The fixed samples were washed three times in an anaerobic 0.05 M cacodylate buffer and then, 1% osmium tetroxide (1–2%, for 1 h) was used for fixing. The samples were then dehydrated with a graded series of ethanol–distilled water mixture (30–100% v/v) and placed in 100% ethanol. The samples were dried by the critical-point drying method before sputter-coating with gold particles. The samples were mounted on aluminum stubs and sputter coated in a Polaron E5100 (VG Microtech), with platinum/palladium target (60:40). The samples were then examined with a JEOL JSM-5800 LV SEM.

Transmission electron microscopy

Samples for analyses with the transmission electron microscopy (TEM) were washed and fixed with 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde for 12–16 h at 4 °C. The fixed samples were rinsed three times (10 min each) at ambient temperature in 0.1 mM

phosphate buffer (pH 7.2) and postfixed with 1% osmium tetroxide (OSO_4) in the same buffer. The samples were then rinsed in 0.1 mM phosphate buffer, and dehydrated through a graded series (30–100%) of ethanol solutions, ethanol–acetone mixture (1:1, for 10 min) and through 100% acetone (two times, 10 min each). Dehydration was followed by sample filtration. A mixture of acetone and spurrs (1:1, for 1 h) and 100% spurrs (for 12–15 h) was used for filtration. Resin was changed prior to embedding. Samples were embedded in Polybed 812 (Polyscience Inc., Warrington, Penn.). Thin sections were cut with Reichert–Jung ultramicrotomes for resin sections and poststained with uranyl acetate and lead citrate. The samples were then examined in a JEOL 1200-EX, which was equipped with digital camera and X-ray microanalysis system.

Cells counting

The TEM micrographs of granules and manure were imported into ArcMap of Geographic Information System (GIS 9.3) software. The grids were overlaid on the micrographs imported in ArcMap. Overlaying the horizontal and vertical grids formed the blocks. Scales of the TEM micrographs were used to calculate the total area of micrographs and the area (nm^2) of micrographs occupied by bacteria. As the grid blocks were square and microorganism's shapes were closer to sphere, a shape factor (0.75) has been used to estimate the area of microorganisms. The respective areas occupied by the microorganisms were estimated based on the three replicate-micrographs for each sample (manure and granules). Reported studies [22, 23] used image analysis techniques to count the cells in samples. Finally cell density was estimated based on the area covered by each group of bacteria (Table 1). Reported microbial population in granule and manure are shown in Table 2 [2, 5, 7]. In this table, % of $(A + B)$ granules represents the individual population percentages in terms of total population of Syntrophobacter and methanogens in granules. The values were calculated as: individual bacteria population divided by the total population of *Syntrophobacter* and methanogens $(A + B)$. Similarly, % of $(A + B)$ manure were calculated to indicate individual bacterial populations [in terms of total populations $(A + B)$] in manure: where A is *Syntrophobacter* and B is methanogens.

Equilibrium modeling

One of the main requirements in equilibrium modeling, that is useful in optimizing the processes [24], is equilibrium data. Anaerobic digestion is considered as an unsteady process. However, considering the usual very low process rates, it is reasonable to assume that equilibrium states were realized within 30 days. Assuming conditions of equilibrium, the Langmuir adsorption model for anaerobic digestion was formulated as Eq. 1 [14, 15]: where, Q_0 is the maximum amount of biogas per g VS of granule (ml of biogas/g VS granule) and K_L represent the amount of manure (g VS) consumed to produce 1 ml of biogas. Q_e (ml of biogas/g VS of granule) and C_e (ml of biogas/g VS of manure) are the gas production at the end of 30, 40, and 50 days. Linearized plot were used to estimate Q_0 and K_L.

Table 1 Population of bacteria in granule and manure samples

Micrograph no.	Total area of micrographs	Area occupied by bacteria	A_b/A_m	Area occupied		Percentage of total area occupied		A_{meth}/A_{syn}
	A_{mc} (nm^2)	A_b (nm^2)		Methanogens A_{meth} (nm^2)	Syntrophobacter A_{syn} (nm^2)	Methanogens	Syntrophobacter	
Granule								
1	65,000	33,375	0.51	18,750	14,625	28.85	22.50	1.28
2	20,533	6,400	0.31	3,700	2,700	18.02	13.15	1.37
3	83,692	29,538	0.35	19,846	9,692	23.71	11.58	2.05
Average	56,409	23,104	0.39	14,099	9,006	24.99	15.97	1.57
SD	32,444	14,593	0.11	9,022	5,992	27.81	18.47	0.42
Manure								
1	65,000	29,625	0.46	3,000	26,625	4.62	40.96	0.11
2	18,667	7,500	0.40	700	6,800	3.75	36.43	0.10
3	43,077	16,385	0.38	5,077	11,308	11.79	26.25	0.45
Average	42,248	17,837	0.41	2,926	14,911	6.92	35.29	0.22
SD	23,178	11,134	0.04	2,189	10,392	9.45	44.84	0.20

A_{meth} and A_{syn} represent the area occupied by methanogens and *Syntrophobacter*

A_{mc} and A_b represent the area of micrographs and total area of bacteria in micrographs, respectively

Table 2 Microbial population in granule and manure

Bacterial groups	Granule[a] (MPN/mL)	Manure (% SSU rRNA)	Percentage of (*A* + *B*) granule	Percentage of (*A* + *B*) manure
Syntrophobacter (A)				
Propionate degraders[b]	3×10^8	1.04[c]	4.68	37.73
Butyrate degraders	1×10^8	0.42[c]	1.52	17.95
Total	4×10^8	1.52	6.24	55.68
Methanogens (B)				
Aceticlastic methanogens[d]	3×10^9	9.59[e]	46.88	7.69
Hydrogenotrophic methanogens	3×10^9	0.19[e]	46.88	36.63
Total	6×10^9	1.21	93.76	44.32

[a] Granular sludge grown on starch industry waste water in mesophilic conditions. Most probable numbers (MPN) was used due to unavailability of 16S rRNA probes data on granule sludge [7, 54]

[b] Sum of *Syntrophobacter fumaroxidans*, *Syntrophobacter pfennigii* and *Syntrophobacter wolinii*

[c] Microbial analysis of inoculum, expressed as percentage of specific SSU rRNA of total SSU rRNA (% SSU rRNA) [5]

[d] Sum of *Methanosarcina* spp. and *Methanosaetaceae*

[e] Methanogens population levels in inoculum, expressed as percentage of specific SSU rRNA of total SSU rRNA (% SSU rRNA) [2]

The Langmuir model [14] can be described as follows:

$$\frac{C_e}{q_e} = \frac{1}{Q_0 K_L} + \frac{1}{Q_0} C_e \quad (1)$$

The extended Freundlich model is the recommended empirical model for processes involving adsorption at a surface because of its desirable statistical properties [25]. The formulation of Extended Freundlich model for anaerobic digestion is given in Eq. 2 [15]. This equation predicted the effect of incubation period on biogas production under different inoculum concentration. In Eq. 2: y is the gas production (in ml) and X is the incubation period (in days). The parameters α, β and γ, are extended Freundlich constants. Constant α and $\beta X^{-\gamma}$ is related to the gas production capacity and intensity of granule, respectively, which describe the influence of inocula (granule) in biogas production from dairy manure. The model evaluates the maximum and minimum values at $X = \exp(1/\gamma)$. The α may be thought of as governing the maximum position of the curve and $\beta X^{-\gamma}$ is a 'shape governing' term [15]. The model was fitted for the gas production data of 7, 12, 15, 18 and 20% inocula concentrations in addition to control treatment. The constant α, β and γ were obtained by fitting model to experimental data of biogas production.

$$y = \alpha X^{\beta X^{-\gamma}} \quad (2)$$

Results and discussion

Microbial structures

Figure 1 shows the scanning electronic micrographs (SEM) and transmission electronic micrographs (TEM) of granules and manure samples. The SEM micrograph (Fig. 1a) shows the granule external structure. The self-immobilization of anaerobic bacteria causes these granules (particulate biofilm) formation in USAB reactors [26]. The size of granules varies (generally 1–3 mm) depending on the wastewater and reactor conditions. The structure and microbial population in the granules increases the anaerobic digestion rate by providing the better interspecies product transfer [27]. The presence of different types of *Syntrophobacter* and methanogens in granules helps in establishing symbiotic relationship necessary to complete the anaerobic digestion [28]. There are several studies that have hypothesized the formation process and performance of granules. A layer model [29] proposed the presence of three types of bacteria in granules. This layer model proposes a central part of the granule consisting of acetoclastic methanogens surrounded by a layer of acetogenic bacteria (layer of hydrogen and formate producing bacteria) and hydrogen and formate consuming methanogens. It has been hypothesized that *Methanosaeta* are the bacteria mainly responsible for initiating granulation [30]. Faster granulation was noted in the presence of *Methanosaeta* [31]. On the surface of granules, fractures, holes, cavities were numerous (Fig. 1a). These micro-pores in the granules could facilitate nutrient transfer throughout the granules. In the surface of granules, the rods representing *Methanosaeta*-like bacteria [9, 32] were abundant. The internal structure of granule (Fig. 1b) shows small plump rods, which resembled *Methanobacterium*-like bacteria (Mbac). These are strictly anaerobes and consume H_2 and/or formate. In general, H_2 and formate act as an electron donor. These bacteria can grow in both mesophilic (37 °C) and thermophilic (48 °C) conditions [33] under

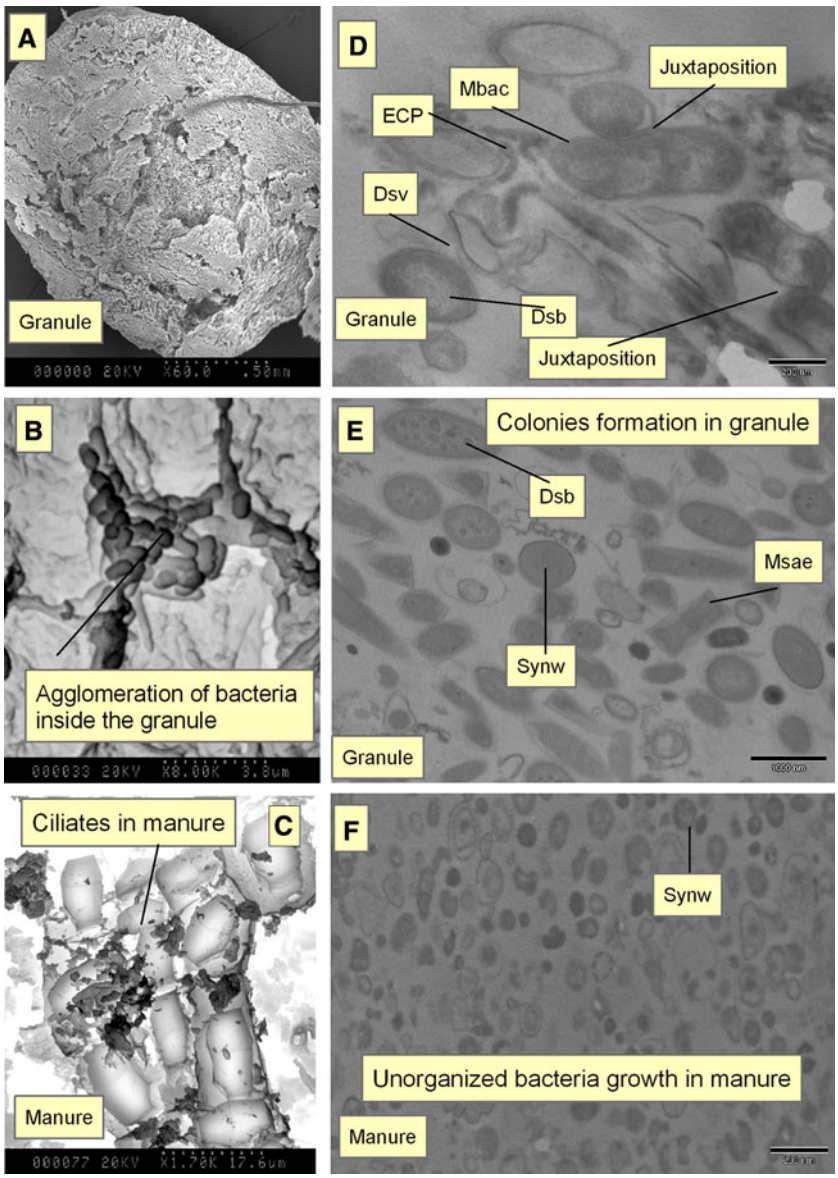

Fig. 1 Scanning electron microscopes (**a**–**c**) and transmission electron microscopes (**d**–**f**) of granules and manure; *Desulfovibrio*-like (*Dsv*), *Desulfobulbus*-like (*Dsb*), *Methanobacterium*-like (*Mbac*), *Methanosaeta*-like (*Msae*), *Syntrophobacter wolinii*-like (*Synw*), and extracellular polysaccharide (*ECP*)

6.0–8.5 pH ranges. The lengths of these bacteria vary widely and are usually in the range of 1–120 μm. *Desulfovibri* (*Dsv*) and *Desulfobulbus* (*Dsb*)-like structures, sulfate reducing bacteria, were also noted in granules (Fig. 1d). Usually, in the absence of sulfate, *Desulfovibri* and *Desulfobulbus* spp. form the syntrophic association with methanogens [34].

Figure 1c, f show the microorganism present in the manure. Usually, dairy manure consists of several types of methanogens, but only a few are more common. The common methanogens in dairy manure are: *Methanobrevibacter*, *Methanomicrobium*, *Methanobacterium*, and *Methanosarcina* [35–38]. The other kinds of microorganisms present in the manure samples were ruminal ciliated protozoa and fungi (Fig. 1c) [39]. Ruminal methanogens utilize reducing equivalents, produced by hydrogen-producing bacteria, anaerobic fungi, and ciliate protozoa [40]. These microorganisms form the unique symbiotic arrangement with other microorganisms. Some other studies have reported that methanogenic bacteria adhere to ciliated protozoa for getting nutrition (acetate and H_2) [41]. Figure 1c shows the several ciliated protozoa-like structures in manure sample.

Figure 1d shows the juxtaposition of *Methanobacter* spp. with *Syntrophobacter* spp. in granule. It is believed that the extracellular polymer (ECP), which is secreted by *Methanobrevibacter* spp. [42] aids bacteria adhesion (Fig. 1d). Several microcolonies of syntrophic acetogens and methanogens were observed within granules (Fig. 1e). The TEM micrograph (Fig. 1e) shows well-structured

microbial colonies in the granules. These colonies consisted of *Syntrophobacter* and *Methanosaeta*-like rods [32]. Other cell structures within colonies were *Desulfovibri* spp., *Methanobacterium* spp., and *Syntrophobacter wolini* spp.-like bacteria. While *S. wolini* spp. are acetogenic bacteria, *Methanosaeta* spp. are considered acetoclastic bacteria. These association between different types of microorganisms and colonies formation helps in anaerobic process by lowing intermediate products such as VFA and H_2. Syntrophic association between *Syntrophobacter* spp. and methanogens provides better interspecies product transfer [7, 9, 43]. Association between VFA producing bacteria (acidogenic) and VFA consuming bacteria (methanogens) quickly consumes VFA and reduces the risk of acidification. Association between H_2 producing acetogenic bacteria and hydrogen consuming methanogens (hydrogenotrophic) reduces the risk of high H_2 concentration. Comparing TEM micrographs of granule and manure (Fig. 1f), manure shows abundant microorganisms, however, the shape and size of these bacteria were different to the bacteria obtained in granule's micrographs. The bacteria present in manure samples were *Methanobrevibacter*-like and *Methanosarcina*-like bacteria. The other structures present in manure were several cist formation, small cocci adhered on fibrous material (not shown in Fig. 1). These cist formations are believed to be formed by *Methanosarcina* [9]. *Methanosarcina* are aceticlastic methanogens, which consume acetic acid and produce methane [2, 44]. Manure micrographs show several methanogens –like structures adhering to protozoa (Fig. 1c). Association of methanogens with protozoa could provide H_2 to methanogens for methane production. The main difference between granules and manure samples (as shown in Fig. 1e, f) was on the distribution of microorganisms. Compared to granules, there were no colonies formation observed in manure samples and also the distribution of bacteria did not have any consistent pattern (Fig. 1f).

Cells density

Table 1 shows the *Syntrophobacter* and methanogens populations in granules and manure samples. The average area of TEM micrographs of granules was 56,409 ($\pm$32,444) nm^2 and the average area of manure's micrographs was 42,248 ($\pm$23,178) nm^2. The average area occupied by bacteria in the granules and manure were 23,104 ($\pm$14,593) nm^2 and 17,837 ($\pm$11,134) nm^2, respectively. Considering the reported population of methanogens and *Syntrophobacter*, it has been noted that there are wide variations among the various reports. The reasons for this could be: the source of granules and manures; technology limitations in identifying the accurate numbers; and human error in detection. Table 2 has been presented to show reported population of methanogens and *Syntrophobacter* in granules and manure. Based on Table 2, granules constitute about 93% methanogens and 6% *Syntrophobacter* and manures constitute 55% *Syntrophobacter* and 44% methanogens. Another study [22], however, reported 46.3% methanogens, and 46.6% *Syntrophobacter* and 7% unknown cells in granules. This study further emphasizes that despite similar color and shape of the granules obtained from different sources, populations of methanogens and *Syntrophobacter* varies greatly with substrate and operating conditions. We used Table 1 (obtained in this study by TEM analysis) to estimate the ratio between methanogens and Syntrophobacter based on the area occupied by bacteria in micrographs. In granule, the *methanogens* and *Syntrophobacte*r percentage were 24.99 and 15.97%, respectively. *Methanogens* and *Syntrophobacter* percentages in manure were 6.92 and 35.29%, respectively (Table 1). The ratio between methanogens to *Syntrophobacter* in granule and manure were 1.57 ± 0.42 and 0.22 ± 0.20, respectively. Considering the variations in the results of this study (Table 1) and reported results (Table 2), further studies using molecular techniques such as fluorescence in situ hybridization (FISH) with 16S rRNA oligonucleotide probes [12] need to be used to provide more precise information on specific microorganisms and heterogeneity of the microorganisms in the granule and manure

Reactor performance

The days of incubation required for onset of biogas production was considered as the reactor start-up period. The gas profiles (Fig. 2a) shows cumulative biogas production in reactors with 7, 12, 15, 18 and 20% of inoculum started on days 4, 4, 4, 3, 2, respectively. No gas production in control reactor was observed until day 8 (Fig. 2a). In reactors with inoculum percentage of 7–15%, the gas production was observed on same day (day 4), while in reactors with 18 and 20% granules, start-up period was reduced to 3 and 2 days, respectively. The earlier onset of biogas in these reactors could be attributed to the structures and associated population of syntrophic and methanogenic bacteria in granules [4, 9, 45, 46].

Figure 2b shows methane composition at various levels of inoculum. The methane content at the onset of biogas production was 40, 42, 42, 55, 58, 55, and 30% on the reactors that received the inoculums 7, 12, 15, 18, 20% and the control, respectively. These results indicate that use of inoculum increased biogas production as well as biogas methane content. The linearized methane content (Fig. 2b) shows that the methane content increased in proportion to the incubation period. The reactor that received 18% inoculums, however, performed poorly and showed

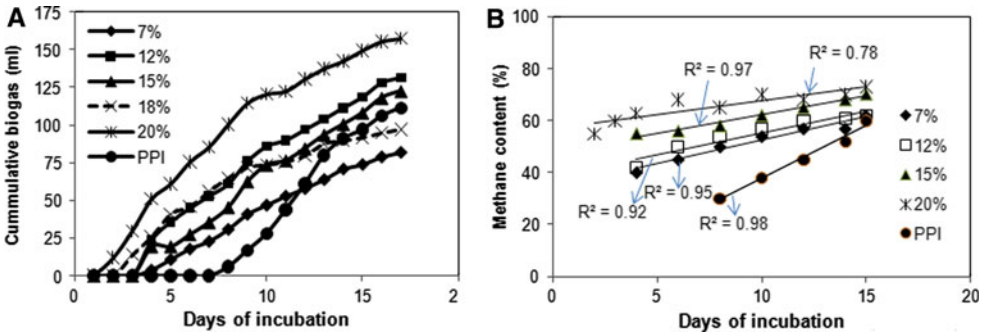

Fig. 2 Cumulative biogas production (**a**) and methane content (**b**)

Table 3 Performances of the reactors

Parameters	Inoculum percentage					
	[6]7%	[3]12%	[2]15%	[5]18%	[1]20%	[4]Control
$Effluent_{,VS}/granule_{,VS}$ (g/g) (F/M)	1.34	0.74	0.57	0.46	0.40	1.00
Methane contents (%) at 15th day	60	64	69	49	74	58
pH	7.02	7.01	7.05	7.23	7.18	7.04
Start-up day	4	4	4	3	2	8
Cumulative gas production over 50 days (ml)	102.5	166.5	170.5	106.5	216.5	162

Mean ± SD

Superscripts in inoculum percentage are performance ranking based on the gas production rate

Population is calculated based on the listed value (Table 1) of *Syntrophobacter* and methanogens in manure and granule. The ratio between of inoculums and manure was used to estimate the combined populations

significantly reduced performance after day 5. In general, while the performances of the other reactors were increasing, this reactor's performance was decreasing in terms of biogas production and methane contents. The poor performance of this reactor may be attributed to the heterogeneity of bacteria in granules and manure. In this reactor poor performance was noticed in first week of incubation which lasted throughout the incubation period. This indicates the importance of start-up period and also demonstrates that inoculating the reactor helped in successful start-up of the anaerobic digestion. It is very important to conduct some preliminary studies using small batch reactors for inocula testing, especially when the intention is to start either a full-scale reactor or a pilot plant. Otherwise, in case of failure, the re-commissioning (emptying the reactor completely and re-starting from using fresh manure) of the reactors can result in unnecessary heavy economic burden. Random inoculation without prior testing the inoculation can lead to reactor failure. Based on the authors' experiences from previous studies, start-up of full-scale reactors with small amount of feed in batch mode could be helpful. In this experiment, disregarding the reactor that received 18% inoculum, the order of (low to high gas production) reactors in terms of gas production in 50 days incubation period were 7, 12, 15, and 20%. The cumulative gas production in control reactor (which did not receive exogenous inoculum) was about same as that for the reactors that received 12 and 15% inoculums. There were differences, however, in terms of the day on which control reactor started biogas production. This is corroborated by previous research in which higher methanogenic populations in initial conditions reduced the delay in biogas production and solid decomposition [4, 47]. Other studies have also emphasized the selection of inocula as critical for anaerobic digestion start-ups. Some studies have demonstrated the influence of inoculum to substrate ratio on biochemical methane potential of maize and stability of the anaerobic digestion process [48, 49]. Both positive and negative effects of different feed/inoculum ratios on the biogas yield have also been reported [50]. In other studies on anaerobic digestion of swine slurry, the use of a correct substrate/inoculum ratio helped in avoiding reactor imbalance [51]. Our study further illustrates two aspects: (1) dairy manure has capability to balance its own microbial consortia after a certain period of time; and (2) if the reactor started successfully, inoculums may not have much influence on increasing the total gas production unless until inoculums concentration is higher (i.e. about 20%). VFA analysis shows that reduced levels of VFA utilizing methanogens resulted in reduced biogas

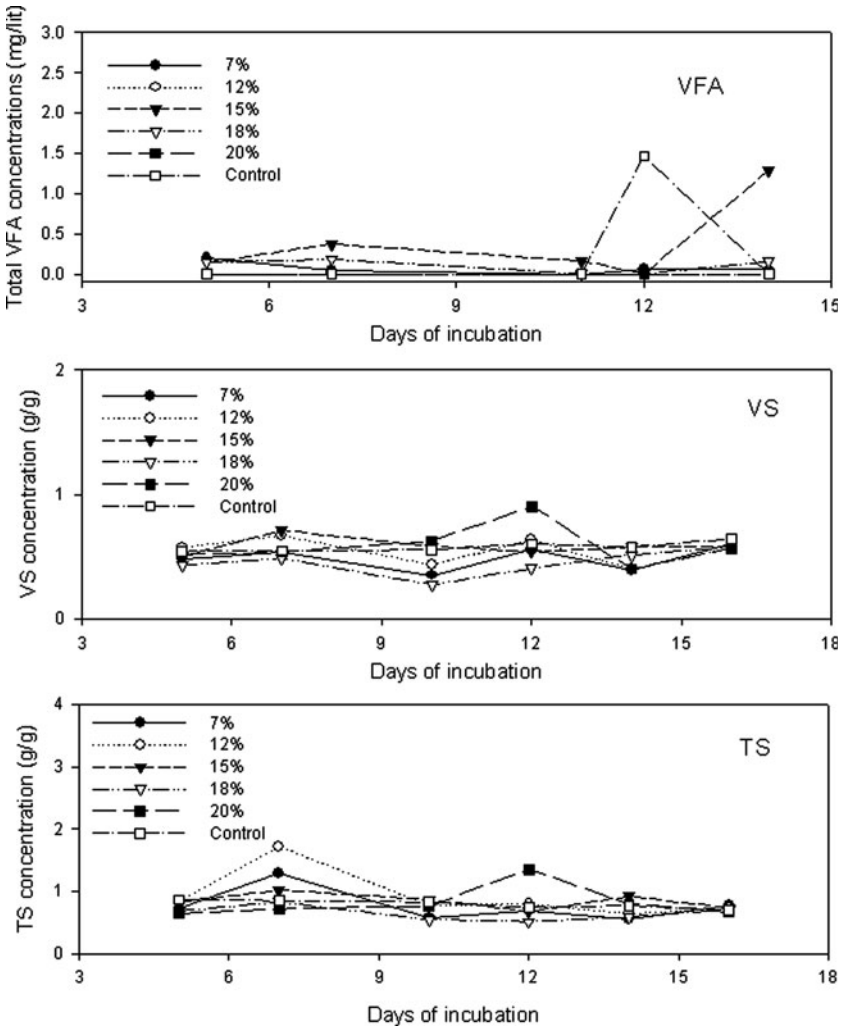

Fig. 3 VFA production, VS and TS reduction with incubation period

production [52]. To reduce the cost of seeding full-scale anaerobic reactors or for practical reasons, the seed may be restricted to 20% or less (may be even 12% to avoid the economic burden). If a reactor treating dairy manure was not seeded with inoculum, longer start-up periods are needed. The onset of gas production in control reactor on day 8 indicates that *Syntrophobacter* and methanogens population was able to balance itself during this period. Table 3 shows the performance ranking of the reactors. The ranking (from higher to lower) was 20, 15, and 12%, control reactor, and 7% of granule in reactor. The higher gas production in control reactor than in the reactor which received 7% of granule indicates exogenous methanogens population in reactor (7% granule) was not enough for increasing the total biogas production although it helped in lowering the start-up period.

The VFA concentrations in the reactors are shown in Fig. 3. The VFA concentrations were low except in one event in the control reactor and one in the reactor that received a 15% inoculum. Since the VFA measurements were sporadic (VFA was not measured daily), it was not possible to establish a relationship between the VFA level and gas production.

The effects of inoculum fraction on TS and VS reduction are shown in Fig. 3. Initially the TS and VS of samples were increased. As the granule had higher TS and VS concentration than the manure, proper mixing of granule with manure and breaking of the granule due to mixing may have raised the TS and VS. The initial increase in TS and VS concentration resulted in increased gas production. The decreased TS and VS concentration on day 11 was followed by decreased gas production. A sudden increase in TS and VS concentrations was observed in reactor that received 20% of granules, which was followed by increased gas production. These results are similar to reported results, which have demonstrated that biogas production rate increases with increase in TS and VS concentration [53].

Table 4 Equilibrium biogas production (q_e) and fraction of biogas produced by granule at different incubation period

Inoculum (%)	Incubation (days)								
	30			40			50		
	q_e (ml/gVS$_{gr}$)	C_e (ml/gVS$_{mn}$)	X_a (%)	q_e (ml/gVS$_{gr}$)	C_e (ml/gVS$_{mn}$)	X_a (%)	q_e (ml/gVS$_{gr}$)	C_e (ml/gVS$_{mn}$)	X_a (%)
7	329.21	119.00	45.41	337.52	116.50	46.56	340.85	115.50	47.02
12	294.85	66.00	69.72	313.28	56.50	74.08	322.97	51.50	76.38
15	228.90	70.50	67.66	242.86	61.50	71.79	264.59	47.50	78.21
20	223.46	26.00	88.07	238.01	13.50	93.81	251.98	1.50	99.31

X_a (%) = 100 × (C_e/C_0); $C_0 = X_0$/g VS of manure for different percentages of manure in mixture; $X_0 = 218$ ml of biogas

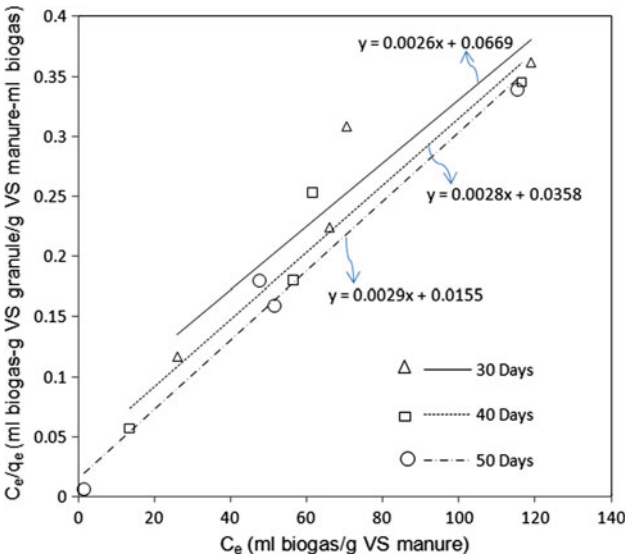

Fig. 4 Linearized Langmuir model plot of biogas production at different incubation period

Modeling results

The equilibrium biogas production and fractions of biogas produced at different incubation periods (30, 40, and 50 days) are presented in Table 4. Based on 50 days cumulative biogas production, most of it was produced within first 30 days of incubation. To estimate equilibrium biogas production (q_e) at different incubation period (30, 40, 50 days; Table 4) for equilibrium modeling, X_0 was taken as 218 ml of biogas, which was the highest cumulative gas production obtained at the end of day 60 of the incubation period, in the reactor that received 20% granule. Linearized plots between C_e (ml biogas/g VS manure) and q_e (ml biogas/g VS granule) are shown in Fig. 4. The Langmuir constants are shown in Table 5. Biogas increased with increase in the incubation period although the rate of gas production decreased after 30 days of incubation while gas production almost ceased beyond day 50 of incubation. The coefficient of determination (R^2) indicates that Langmuir equation could be useful in modeling the start-up of the reactors. R^2 values for 30, 40, and 50 days incubation period were 0.88, 0.95 and 0.98, respectively (Table 5).

Table 5 Langmuir constants and coefficient of determination

Incubation (days)	Langmuir constants		
	Q_0 (ml biogas/g VS granule)	K_L (g VS manure/ml gas)	R^2
30	384.62	0.039	0.88
40	357.14	0.078	0.94
50	344.83	0.193	0.98

The extended Freundlich model using SAS 9.1 (SAS Institute Inc., Cary, NC) was fitted to the biogas production data. The model predictions compared well with the measured values for all reactors. The result of extended Freundlich model prediction is shown in Fig. 5 for the reactor that receives 12% inocula. The extended Freundlich equation shows very good fit when predicted and measured cumulative biogas production was plotted against the incubation period (Fig. 5). Linearized plots between predicted biogas production (ml) and measured biogas production (ml) are shown in Fig. 6. Table 6 shows the extended Freundlich constants (α, β and γ), statistical results, and R^2 values. Based on the coefficient of determinations, we concluded that extended Freundlich model is suitable for modeling the effects of incubation period on cumulative biogas production and hence the start-up of the anaerobic process. A comparison between measured and predicted gas values indicated that the predicted cumulative gas production was 4.5, 5.5, 4.4, and 5.3% higher than the measured gas production in reactors receiving 7, 12, 15, and 20% of granule, respectively. In the control reactor, a predicted gas value was 2% lower than the measured values. Because these studies were based on batch-fed reactors (maintained at a constant temperature (35 °C) with no feed change during incubations), the results can only be

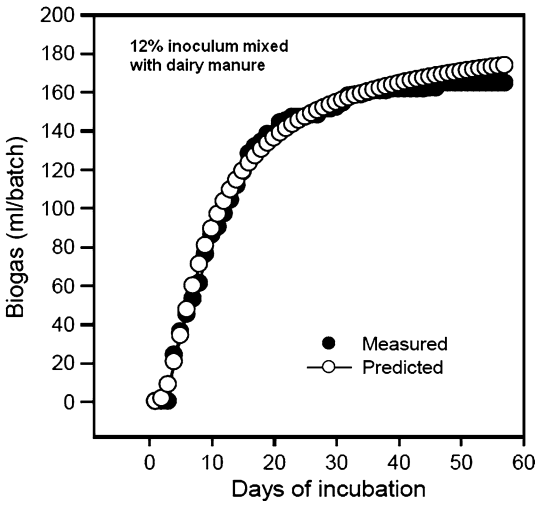

Fig. 5 Extended Freundlich model plot of biogas production

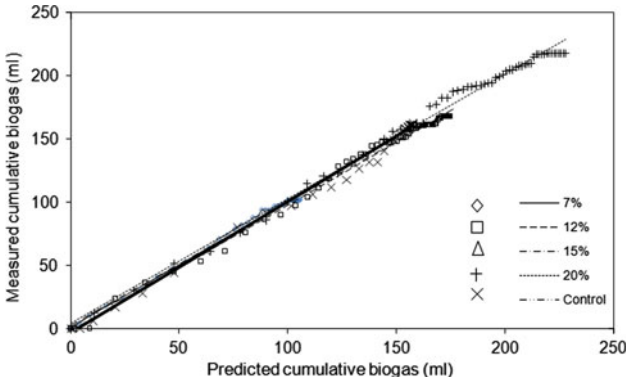

Fig. 6 Linear plot of extended Freundlich model (measured vs. predicted)

useful for predicting the start-up of batch anaerobic digestion process, which is usually the way all anaerobic processes are started.

Conclusions

This study demonstrates that the proportion of inoculum is important for successful start-up of anaerobic digestion process. The SEM and TEM micrographs are helpful in identifying the bacteria communities present in the granules and the manure. A vast majority of microorganisms was observed in both the granules and the manure samples. The equilibrium modeling indicates that the Langmuir equation and extended Freundlich equation are suitable for designing and optimizing the start-up of anaerobic processes.

Table 6 Extended Freundlich constants and coefficient of determinations

Inoculum	Constants												
	α				β				γ				
	Estimate	Std error	Lower confidence limit 95%	Upper confidence limit 95%	Estimate	Std error	Lower confidence limit 95%	Upper confidence limit 95%	Estimate	Std error	Lower confidence limit 95%	Upper confidence limit 95%	R^2
7%	1.1×10^{-6}	2×10^{-6}	-3×10^{-6}	5.9×10^{-6}	21.10	2.68	15.70	26.54	0.49	0.010	0.47	0.51	0.995
12%	0.0020	0.0040	−0.006	0.0170	13.82	2.35	9.10	18.54	0.59	0.028	0.53	0.65	0.994
15%	0.0003	0.0008	−0.001	0.0020	14.53	3.17	8.17	20.88	0.51	0.020	0.46	0.56	0.993
20%	0.2380	0.1348	−0.032	0.5084	9.54	0.95	7.62	11.46	0.80	0.040	0.71	0.88	0.992
Control	1×10^{-25}	1×10^{-24}	-2×10^{-26}	2×10^{-24}	65.63	8.40	48.77	82.48	0.40	0.003	0.39	0.41	0.996

* $p < 0.05$

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