A novel enhancement for the start-up of methane fermentation reactor by inoculating the acclimated sludge as a seeding material

N. T. Dinh*

Research Institute for Sustainable Development, Hochiminh City University of Natural Resources and Environment, 236B Le Van Sy, Ward 1, Tan Binh District, Hochiminh City, Vietnam

*dtnga@hcmunre.edu.vn

Abstract: This study aimed to evaluate the strategy for accelerating the start-up of anaerobic reactor treating glycerol. The experiment was carried out for 25 days duration in two cases: (1) gradually increase of glycerol content in substrate from 33% to 67% to 100% after 13 days (runs: G1 and B1); (2) feeding with 100% glycerol content in substrate from the start (runs: G2 and B2). G1 and G2 were inoculated by one third of acclimated granular sludge to treat glycerol and two third of granular sludge taken from a brewery wastewater treatment system. In results, the average methane production rate (L CH₄ L⁻¹ day⁻¹) of runs G1; B1; G2; B2 was 0.56; 0.39; 0.42; and 0.14, respectively. G1 was the best performance among runs. G2 and B1 were unstable during the start-up. B2 was fluctuant throughout the duration time. The methane production rate of G1 and B1 was highly correlated and DNA concentration of Genus Trichococcus in the sludge. This study suggests that gradual feeding substrate during start-up could provide a stable performance compare to the shock feeding. Acclimated sludge which contained functional microorganisms of treating glycerol could accelerate the start-up of new anaerobic digester.

1. Introduction

Anaerobic digestion is the consequence of many metabolic interactions of anaerobic microorganisms in the absence of oxygen [1]. The overall process converts biodegradable substrates to biogas through successive reactions. Large, complex soluble or insoluble compounds are broken down, through hydrolysis, into smaller molecules. In the next step, acidogenesis, the simpler or smaller molecules are converted to alcohols and volatile fatty acids (VFAs) such as acetate, butyrate and propionate. The larger organic acids are further converted to acetate, carbon dioxide and hydrogen. Some of the microorganisms involved in this latter step are hydrogen-producing acetogenic microorganisms. Finally, acetate is converted to methane by acetotrophic methanogenic microorganisms and carbon
dioxide and hydrogen are converted to methane by hydrogenotrophic methanogenic microorganisms [2-4].

Methanogens, which are archaea, are more sensitive to growth conditions and grow much slower than the acidogetic bacteria [5]. Consequently, VFAs and other intermediate substances, such as hydrogen, tend to have higher levels during start up as they are not readily converted by the slow methanogenesis. When VFA level builds up, pH may drop to inhibitory levels for methanogens, thereby, further inhibiting the process. When hydrogenotrophic methanogens are inhibited, the partial pressure of hydrogen may reach inhibitory levels (>10^{-4} atm) for the preceding step, e.g., acetate- and hydrogen-producing beta-oxidation [6]. To avoid this phenomenon, a balanced population of various functional microbial groups (bacteria and archaea) must be achieved and maintained [3, 4, 7].

Although there are several microorganisms known to degrade glycerol but the glycerol seed sludge source for start up of industrial-scale methanogenic reactor is uncertain. Previous studies indicated that glycerol is more difficult to degrade than glucose, lactic acid, and starch. Because it requires to convert glycerol to other intermediates such as glycerol-3-phosphate (G3P), dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) before it can join the common pathways of anaerobic digestion for further conversion [8, 9].

The start-up of new anaerobic digester treating recalcitrant substrate is challenge. It requires for long time and has high potential of deterioration. The inoculation of the seed sludge from a methane fermentor, which has been operated stably, is often applied to accelerate the start-up of a new methane fermentor, even if the types of substrate in the previous and the new fermentors differ from each other. If the seed sludge is introduced into a fermentor treating different types of substrate, a microbial community may change with the progress of the acclimation stages from the previous substrate to the new substrate [10]. Therefore, it is necessary to investigate strategies for glycerol methane fermentation in order to achieve the good reactor performance and prevent from substrate inhibition or overloading. Previous studies, some authors investigate about methane fermentation of glycerol but they did not focused on the acclimation of glycerol to the substrate [11],[12],[13]. López, Santos [12] investigated about anaerobic digestion of glycerol. Before during the experiment with glycerol, the solution of glucose, sodium acetate and lactic acid was fed to the reactor, after at glycerol was gradually replaced the original substrate by increasing the percentage of glycerol used in the from 25% to 100% after four loads while maintaining COD concentration. Nevertheless, this research did not indicated about the functional of acclimation. Vásquez and Nakasaki [14] investigated about the effects of shock loading versus step wise acclimation on microbial community during the anaerobic digestion of glycerol. They found out that the different feeding methods of glycerol led to different functional bacterial community for methane fermentation. However, both shock loading and step wise loading could achieve similar methane production rate from glycerol. However, they have not investigated about the role functional bacterial community for methane fermentation in enhancing start-up of new anaerobic reactor treating glycerol.

In the previous study, we worked on the changes in bacterial and archeal community during anaerobic digestion of glycerol and subsequent methane fermentation. A laboratory-scale repeated-batch methane reactor was used for the acclimation of seed sludge from a full-scale methane reactor treating brewery wastewater to treat glycerol. Glycerol concentration was gradually increased during the acclimation period. As a result, the dynamic change of microbial consortium corresponded to the changes in the types of substrate (from brewery wastewater to glycerol). The quantification of
microbial community elucidated that the functional microorganisms for anaerobic digestion of glycerol are genus *Trichococcus* and family *Syntrophomonadaceae* (for bacteria) and genera *Methanobacterium* and *Methanospirillum* for archaea [10].

In the present study, we expected to accelerate the start-up of new anaerobic reactor treating glycerol by using the acclimated seed sludge of our previous study. The overall aim of this study is to determine start-up strategies for enhancing the start-up of glycerol methane fermentation process.

2. Materials and methods

2.1. Seed sludge composition

Two types of granular sludge were used as seed sludge: (1) Brewery granular sludge (BGS) that was harvested from a full scale of UASB reactor treating brewery wastewater; (2) Glycerol granular sludge (GGS) that was obtained from an anaerobic sequencing batch reactor (ASBR) in which granular sludge was acclimated for treating glycerol in two months. Our previous study elucidated the contribution of genera *Trichococcus*, *Methanobacterium* and *Methanospirillum* were functional microorganisms for methane fermentation of glycerol [10]. These genera were dominant microorganisms and present at high concentration in GGS (Refer figure. 1).

![Figure 1. Log Relative DNA concentration of dominate microorganisms in glycerol granular sludge.](image)

2.2. Experimental organization

In order to clarify the function of acclimated granular sludge in accelerating the start-up of new bioreactor, four runs of experiment were carried out in two cases (Refer table. 1 and figure 2). In total 300 mL of seed sludge, the portion was 33% of GGS plus 67% BGS for runs G1&G2; 100% BGS for runs B1&B2.
Figure 2. The schematic of experimental organization.

For carbon sources, in run G1 & B1 the percentage of glycerol in the influent was gradually increased from 33% (from day 1 to 6) to 67% (from day 7 to 12) and 100% (from day 13 to 25) in term of COD; the other portion or carbon source was glucose, sodium acetate, and lactic acid (GAL); In run G2 & B2 the glycerol content in the influent was suddenly fed with high concentration (100%) at the start-up. The effect of acclimated granular sludge in enhancing the performance and reducing the start-up time of new reactor was achieved by comparing the performance of these runs.

Table 1. The substrate and seed sludge composition of two feeding strategies.

| Run name | Seed sludge type portion | Substrate composition (% of COD) |
|----------|--------------------------|----------------------------------|
|          |                          | Days: 1-6                        | Days: 7-12                        | Days: 13 - 24                        |
| G1       | 33% GGS + 67% BGS        | 33% Glycerol; 67% Glycerol;       | 67% Glycerol; 33% GAL              | 100% Glycerol                        |
| B1       | 100% BGS                 | 33% Glycerol; 67% Glycerol;       | 67% Glycerol; 33% GAL              | 100% Glycerol                        |
| G2       | 33% GGS + 67% BGS        | 100% Glycerol                    | 100% Glycerol; 100% Glycerol       | 100% Glycerol                        |
| B2       | 100% BGS                 | 100% Glycerol                    | 100% Glycerol; 100% Glycerol       | 100% Glycerol                        |

2.3. Reactor and operation
Four 3-L working volume reactors made of Pyrex glass (250 mm in height and 120 mm ID) were used. Each run was carried out in 25 days duration. Reactors were operated in successive 24 h cycles consisting of 2h effluent (750 mL) withdrawal and influent (750 mL) filling, 19.5 h reaction period, and 2.5 h settling. The 750 mL of supernatant liquid withdrawn from the reactor was subjected to
physicochemical and biological analyses. The contents of the reactor were mechanically agitated at 100 rpm, and the reactor itself was kept in a water bath to maintain the temperature at 39 °C. A sample of the exhaust gas, i.e., biogas, was captured in a Tedlar bag. The biogas volume was measured daily using a dry test gas meter (DC-1, Shinagawa, Tokyo, Japan). The composition of the biogas was analyzed using gas chromatography-mass spectrometry (GC-MS). The methodology for analyzing other parameters such as ORP, pH, VFA were described detail in previous paper [10].

2.4. Molecular analysis of microbial communities

Five sludge samples were taken during the operational period and further analyzed for microbial quantity. The ISOIL for Beads Beating Kit (No. 319-06201, Nippon Gene Co., Ltd., Toyama, Japan) was used to extract the DNA from 0.3 g wet weight granular sludge sample by following manufacturer’s instructions. The extracted DNA was used as a template for PCR. PCR was performed using the TaKaRa ex Taq kit and an automated thermal cycler (PCR thermal cycler dice, TaKaRa, Shiga, Japan). Primers that amplify the 16S rRNA gene for bacteria were follows: forward primer 357FGC [5' - CGC CCG CCG CGC GCG GCG GGC GGGGCG GGGGCA CGG GGGGCCTACGGGAGGCAG CAG- 3'] and reverse primer 517R [50 - ATTACCGCGGCTGCT GG- 3'] [15]. The PCR product was used for denaturing gradient gel electrophoresis (DGGE), cloning and DNA sequencing to identify the functional microorganism, the procedure of these steps was described previously [10]. The sequences of DNA were compared to the sequences of the 16S rRNA genes available in databases (DDBJ, EMBL and GenBank) in order to determine the microorganisms present in the experiments.

Real-time PCR was performed to quantify the bacteria found to be dominant in the sludge samples in both glycerol-loading approaches. The primer sets used for the real-time PCR analysis and the conditions and equipment used for the real-time PCR were as described previously [10]. In Real-time PCR reaction, Ct values were obtained for each sample. The Ct value shows the number of reaction cycles required to reach the threshold value of the signal obtained during the real-time PCR and is inversely proportional to the DNA concentration of the samples. The corrected relative cell density of each bacterium was calculated using the following equation:

\[
RC_i (t) = \frac{2^{Ct_R (t=T)} - C_i (t)}{2^{Ct_i (t=T)} - C_{max} i}
\]

RC_i (t) indicates the corrected relative cell density of a species of bacterium, i, at a certain time, t. C_i (t) and C_{max} i represent the cell density of a species of bacterium, i, calculated from the calibration curve at a certain time, t, and the maximum cell density during fermentation, respectively. C_{t_R} (t=T) and C_{t_i} (t=T) represent the Ct value of the real-time PCR for the reference bacterium and species of bacterium, i, respectively, at the fermentation time, T.

3. Results and discussion

3.1. pH, ORP

During the experimental period, pH value of all runs was maintained at neutral range to make good condition for the growth of microorganisms [11], [10]. The ORP values of G1; B1; G2; and B2 at the first day of experiment were -320 mV and -200 mV, -320 mV; and -320 mV, respectively. After that, ORP decreased sharply in all runs and reached around -400 mV after 4 days operation and ORP profiles were almost stable at this value during the experiment period of all four runs (data are not shown). It means that the anaerobic condition was maintained throughout the whole duration of experiment which is similar signal of ORP that indicated in previous studies [10], [16].
3.2. Average methane production rate (MPR) and average methane yield percentage (MYP)

During the experimental period of the runs, the biogas production was evaluated and calculated of methane production rate. It was shown that run G1 was the most stable during time course and achieved highest MPR among the runs, MPR of run B1 and G2 was low at the start and getting stable in the later phase, run B2 performed unstably throughout the operated time. The average methane production rate (L CH₄/L/day) of run G1; B1; G2 and B2 was 0.56; 0.39; 0.42; and 0.14, respectively (Refer table 2).

Table 2. The average methane production rate and methane yield during time course.

| Run name | Seed sludge type portion | Average Methane production rate (L CH₄ L⁻¹ day⁻¹) | Average Methane yield percentage (%) |
|----------|--------------------------|-----------------------------------------------|--------------------------------------|
| G1       | 33% GGS + 67% BGS        | 0.56                                          | 62.17                                |
| B1       | 100% BGS                 | 0.39                                          | 44.65                                |
| G2       | 33% GGS + 67% BGS        | 0.42                                          | 48.00                                |
| B2       | 100% BGS                 | 0.14                                          | 16.09                                |

Figure 3. The comparison of methane yield percentage. (a) Runs G1 vs B1. (b) Runs G2 vs B2.

From the methane production rate of each reactor, methane yield was calculated as the percent of organic matter from the influent substrate converted to methane. In the same trend of MPR, the average of MYP of for runs was 62.17 % for G1; 44.65% for B1; 48.00% for G2 and 16.09% for B2 (Refer table 2). For more detail of MYP, the methane percentage of runs G1 vs B1 and runs G2 vs B2 is illustrated in figure 3. It was indicated that the methane yield of G1 was much higher than that of
B1 during the start-up period. From day 13 of the experimental period, these reactors were fed with 100% of glycerol, the difference of methane yield between G1 and B1 was not high. It can be attributed that when glycerol was fed with low concentration at the start-up and gradually increased during the time course, the function of acclimated granular sludge could express their effect from the early stage, however the without acclimated granular sludge the reactor B1 could overcome the start-up and performed better in the latter stage. When glycerol was feed with high organic loading rate during the start-up the effect of acclimated granular sludge was demonstrated clearly. Namely, in run G2, the methane yield was low in the early stage after that it was improved and reached 65% in the middle of the time course. Whereas the performance of B2 was extremely fluctuated throughout the time course and methane yield was very low (less than 20%). The much difference of methane yield between runs G2 and B2 elucidated clearly the effect of acclimated glycerol granular sludge.

3.3. Volatile fatty acids (VFAs) concentration

The VFAs concentrations in the effluent of four runs are illustrated in figure 4. The general trend in VFAs profiles show that the VFAs concentration of run G1 was lowest among four runs throughout the whole experiment. These VFA profiles corroborate the high biogas production rates resulting high methane yield. This indicated that the VFA accumulation did not happen in this run. These data indicated a stable performance of G1 throughout the progress of anaerobic digestion. In run B1 and run G2, high values of total VFAs concentration was observed during the first two weeks of the experiment, and getting decreased in the later phase. The high concentration of VFAs indicates weak performance of the reactor during the first two weeks of experimental duration. After the start-up period, the performance of runs B1 and G2 were improved and become stable as indicated by the better methane yield percentage. The VFAs concentration was also stable and decreased significantly. Perhaps, the microbial community was shocked at the early period of start-up and as they were continuously exposed to the substrate and the intermediate degradation products, they gradually became adapted to glycerol and overcome the imbalance situation that occurred in the early stage of the experiment.

Run B2 was fed with the same organic loading rate as other runs and the maximum of total VFAs concentration was very low that was 1.3 g/L at day 5 and decreased to under 0.4 g/L until day 23 of the experiment. However, methane yield percentage was very low throughout the whole duration of run B2. The relationship between VFA and gas production in run B2 compared to that of other runs indicates that microorganisms for all steps of anaerobic digestion in run B2 were inhibited thus they could not utilize glycerol and convert it to VFAs and they could not convert all VFAs from glycerol to methane.

The difference in VFAs accumulation concentration in four of these runs can be attributed to the seed sludge composition because it is the unique distinguish point for these reactors. Runs G1 and G2 were inoculated by one third of seed sludge amount is acclimated glycerol granular sludge that contains functional microorganism for treating glycerol. The present of dominant bacteria (genus *Trichococcus* and genus *Syntrophomonadaceae* and family *Syntrophomonadaceae*) and dominant archaea (genus *Methanobacterium*; and genus *Methanospirillum*) for glycerol utilization perhaps they were achieved the stable condition rapidly thus the VFA production and VFA conversion to methane was balance thus the VFA concentration of this reactor was very low. A previous study also found that the family *Syntrophomonadaceae* is a phylogenetically distinct group that generally grows in a syntrophic association with hydrogenotrophic methanogens, such as the genera *Methanospirillum* [17] and *Methanobacterium* [18], in the methane fermentation of long-chain fatty acids. Whereas, run G1 was operated with the seed sludge that harvested from the reactor treating brewery wastewater, the seed sludge did not contain the microbial community that suitable for treating glycerol. Therefore, when reactor was started-up with glycerol as one of carbon sources the imbalance condition occurs, and methanogens was inhibited thus they could not convert VFAs to methane resulting in VFAs accumulation.
Figure 4. Volatile fatty acid concentration profile for four runs: (a) Run G1; (b) Run G2; (c) Run B1; (d) Run B2.
The VFA accumulation occurred in the first two weeks of the experiment period of G2 when high glycerol loading rate was fed since the start-up time however, it could overcome and the performance of this run was improved in the latter two week. The improvement of G2 was attributed to the functional microorganisms contained in acclimated glycerol sludge in the seed sludge composition. Whereas, without glycerol functional microorganisms inoculated to B2, this run displayed weak performance and fluctuation throughout the time course.

Gallert and Winter [6] found out that the anaerobic digestion process was disturbed successively in different stages if hydrogen accumulated due to an overloading or to inhibition of methanogen. Firstly, in the acetogenic stage of oxidation of fatty acids and alcohols failed, leading to an accumulation of these acid metabolites. After that, the spectrum of metabolites of the fermentative flora changed toward more reduced products like ethanol, lactate, propionate, and n-butyrate, leading to an even higher concentration of volatile fatty acids. Siegert and Banks [19] elucidated that VFA concentrations above 2 gL⁻¹ caused to inhibition of cellulose degradation. The fermentation of glucose was slightly inhibited at VFA concentrations above 4 gL⁻¹. Wang, Zhang [20] the inhibition in the activity of methanogenic bacteria was not occurred then the concentrations of ethanol, acetic acid and butyric acid reached 2400, 2400 and 1800 mg L⁻¹, respectively.

The VFAs concentration during time course of these four runs confirmed the contribution of functional microorganisms in seed sludge to the performance of anaerobic digester. A suitable microbial community for a specific substrate could accelerate the startup time and maintain a stable performance for reactor. Otherwise, when the microbial community is unbalance and not suitable with the target substrate it could make the VFAs accumulation and failure performance.

3.4. The relationship between methane production rate and cell density of functional microorganisms

It was indicated in our previous study that Trichococcus was dominated genus among the bacteria community during the acclimation stages of the methane fermentation for the treatment of glycerol [10]. The present study, quantity of this genus during time course for run G1 and run B1. Figure 5 shows the correlation between methane production rate and DNA concentration of Genus *Trichococcus*, it is clearly to see that in the run G1 that seeded by glycerol granular sludge, the DNA concentration of Genus Trichococcus was high at the start and maintained stably in the whole of experimental duration. Otherwise, DNA concentration was low at the start and getting increase during time course. Interestingly, the dynamic *Trichococcus* concentration was same trend of methane production rate (red line for run G1 and violet line for run B1). The relation between methane production rate and DNA concentration of Genus *Trichococcus* in run G1 and run B1 could clearly demonstrated the role of functional microbial community in performance of anaerobic digester. Some previous studies also mentioned about this relationship. From another study, Yang, Phan [21] also found out that the richness hydrolysis and acetogenesis microorganisms of *Bacteroidales* and *Syntrophobacterales* decreased the biogas production from anaerobic digester was decreased. Recently, there are not many researches those investigated about the relationship between biogas production rate and quantity of functional microorganisms in anaerobic digester.
Figure 5. The correlation between methane production rate and DNA concentration of Genus *Trichococcus*.

4. Conclusions
Glycerol granular sludge (GGS) which contained functional microorganisms of treating glycerol could accelerate the start-up of new anaerobic digester treating glycerol in both step wise feeding and shock feeding. The methane production rate (MPR) had close relation with and DNA concentration of Genus *Trichococcus* in methane fermentation of glycerol. Namely, the MPR changed in the same trend with the concentration of functional microbial community of anaerobic digester. The gradual feeding of glycerol substrate during startup could provide a stable performance compare to the shock feeding of glycerol for methane fermentation system. The shock feeding of glycerol to the reactor inoculated by brewery granular sludge could cause the deterioration of methane fermentation digester.

Acknowledgments
The author would like to impress her sincere thanks to Prof. Analiza P. Rollon, Department of Chemical Engineering, University of the Philippines, Diliman, Philippines and Prof. Kiyohiko Nakasaki, Department of International Development Engineering, Tokyo Institute of Technology, Japan for advising and accommodation the experimental facilities.

References
[1] Abudi Z N, Hu Z, Sun N, Xiao B, Rajaa N, Liu C and Guo D 2016 *Energy*. 107 131-40
[2] Liu W-T, Chan O-C and Fang H H 2002 *Water Res.* 36(13) 3203-10
[3] Pandey P K, Ndegwa P M, Soupir M L, Aldredge J R and Pitts M J 2011 *Biomass Bioenergy*. 35(7) 2705-20
[4] Nakasaki K, Kwon S H and Takemoto Y 2015 *Biomass Bioenergy*. 78 17-24
[5] Dong F, Zhao Q-B, Zhao J-B, Sheng G-P, Tang Y, Tong Z-H, Yu H-Q, Li Y-Y and Harada H 2010 *Bioresour. Technol.* 101(6) 1722-26
[6] Gallert C and Winter J 2005 Bacterial metabolism in wastewater treatment systems. Environmental Biotechnology. Concepts and Applications (Germany: Wiley-VCH) 1-48
[7] Lins P, Reitschuler C and Illmer P 2012 Bioresour. Technol. 110 167-73
[8] Barker D J and Stuckey D C 2001 Water Environ. Res. 73(2) 173-84
[9] Nuchdang S and Phalakornkule C 2012 J. Environ. Manage. 101 164-72
[10] Dinh N T, Hatta K, Kwon S H, Rollon A P and Nakasaki K 2014 Biomass Bioenergy. 68 240-49
[11] Yang Y, Tsukahara K and Sawayama S 2008 Process Biochem. 43(4) 362-67
[12] López J Á S, Santos M d l Á M, Pérez A F C and Martín A M 2009 Bioresour. Technol. 100(23) 5609-15
[13] Astals S, Nolla-Ardèvol V and Mata-Alvarez J 2012 Bioresour. Technol. 110 63-70
[14] Vásquez J and Nakasaki K 2016 Biomass Bioenergy. 86 129-35
[15] Ueno Y, Haruta S, Ishii M and Igarashi Y 2001 Appl. Microbiol. Biotechnol. 57(1-2) 65-73
[16] Appels L, Baeyens J, Degrève J and Dewil R 2008 Prog. Energy Combust. Sci. 34(6) 755-81
[17] Zhao H, Yang D, Woese C R and Bryant M P 1993 Int. J. Syst. Evol. Microbiol. 43(2) 278-86
[18] Sousa D Z, Smidt H, Alves M M and Stams A J 2007. Int. J. Syst. Evol. Microbiol. 57(3) 609-15
[19] Siegert I and Banks C 2005 Process Biochem. 40(11) 3412-18
[20] Wang Y, Zhang Y, Wang J and Meng L 2009 Biomass Bioenergy. 33(5) 848-53
[21] Yang S, Phan H V, Bustamante H, Guo W, Ngo H H and Nghiem L D 2017 Bioresour. Technol. 234 439-47