The effect of different sludge pretreatment methods on mitigation of anaerobic digester foaming

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Abstract

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In this study, waste activated sludge obtained from two full-scale treatment plants with foaming issues was pretreated with acid/alkali treatment, acid-phase fermentation, thermal treatment, sonication, and metal salt treatment to investigate their effect on foam mitigation. The major foam forming organisms were identified by microscopy and qPCR as *Gordonia amarae*, which are characterized as branched filaments. Waste activated sludge was characterized for foaming index prior to each pretreatment method. After carrying out different pretreatment methods, the foaming potential was again measured in batch experiments carried at different holding times per pretreatment methods. Among all pretreatment methods investigated, acid/alkali treatment and acid phase fermentation both showed a high reduction in foaming and in inactivation of *G. amarae* filaments and thermal treatment combined at alkaline pH enhanced the effectiveness of foam reduction. The maximum foaming reduction of 56% was achieved upon holding the sample for 2.5 h at 70ºC and pH of 11. Pretreatment methods that resulted in a sharp decrease in the foaming potential concurred with numbers in dead foam formers as confirmed using live/dead staining and the PMA-qPCR technique. Pretreating with FeCl3 gave good foaming reductions with 53.5% reduction in the unstable foaming potential at a concentration of 260g/L but did not result in a decrease of foam formers as confirmed by live/dead staining. Sonication did not prove effective in lowering the foaming index or killing the *G.amarae* filaments.

Keywords: Filamentous bacteria, anaerobic digester foaming, *Gordonia amarae*, pretreatment, PMA-qPCR, live/dead stain

Introduction

Anaerobic digestion consists of a series of biological reactions that includes hydrolysis, acidogenesis, acetogenesis and methanogenesis that convert biodegradable feed solids to biomethane gas which can be utilized for energy generation [4]. Different methods have been developed over the last century to increase the biogas yield including thermal hydrolysis [24], acid pretreatment [7], alkali pretreatment [17] and acid-phase digestion [6]. The basic principle of all these pretreatment methods is to enhance the first step of anaerobic digestion (hydrolysis) thereby making more organic carbon available for methanogens and hence increasing the biogas production. The daily biogas production volume can be more than 15 times the feed volume and in some cases the gas production leads to anaerobic digesters foaming. While there is a lot of literature investigating the advantages of pretreatment methods on biogas production, examinations on studying the benefits of sludge pretreatment on the mitigation of foam formation in anaerobic digesters are sparse [9]. Digester foaming can be a serious issue leading to overflowing digesters, fouling of gas compressors and pipework, odor issues, and decreased biogas production. Whether a digester foams or not depends on several factors such as VFA production, pH, temperature, sludge retention time, surface characteristics, nature and type of surfactants, and the CO2:CH4 ratio [23][25]. Foaming is a three-phase phenomenon between surfactants (solids), sludge water and biogas [23]. Surface properties of all three constituents determine how stable the foam is. Subramanian et al. [23] suggest that while surfactants are responsible for causing foaming, the filamentous bacteria present in the waste activated sludge help stabilize the foam by preventing drainage of the liquid film around gas bubbles (Figure 1). Jenkins and coworkers explained that even a small concentration of filaments present in the activated sludge can cause foaming in anaerobic digester since these filaments are much more concentrated in the waste activated sludge that is being fed to the digesters [21]. The two most commonly found filaments in foaming sludge are *Gordonia amarae* and *Microthrix Parvicella* [14][20]. There have been several theories about how filaments contribute to digester foaming. Filamentous bacteria belonging to genus *Gordonia* have a hydrophobic cell surface which is composed of mycolic acid [14] through

which they get attached to the gas bubbles and cause stable foam in digesters. Another theory suggests that if filaments reach concentrations higher than their critical micelle concentrations, they form web like structures, binding the flocs together resulting in a poor settling sludge and an increased surface activity, thus contributing to surface foaming [20]. Some measures of decreasing the growth of foam forming bacteria include elevating the process temperature (thermophilic treatment) [16], surface wasting and increasing the food to mass ratio as it favors the growth of *Gordonia amarae* [8]. Additionally, sludge pretreatment methods that are used to increase biogas yield in anaerobic digesters might also be effective in decreasing the foaming potential. So far, only a few studies have been reported of using pretreatment methods to decrease digester foaming [1][12][9] but in all these studies neither systematic tests directed at finding optimal conditions for foam reduction were conducted nor were different pretreatment methods compared for their effectiveness in decreasing digester foaming. In this study, we have used different sludge pretreatment methods such as acid/alkali treatment, ultrasonication, thermal treatment, metal salt treatment and acid-phase fermentation to test their impact on reducing the foaming potential of the waste activated sludge of anaerobic digesters. The impact on the microbial population was followed using live/dead staining in addition to PMA-qPCR to quantitatively screen for the efficiency of each pretreatment method to kill the foam-forming *G. amarae* bacteria and to evaluate the linkage of reduction in filaments to foaming potential.

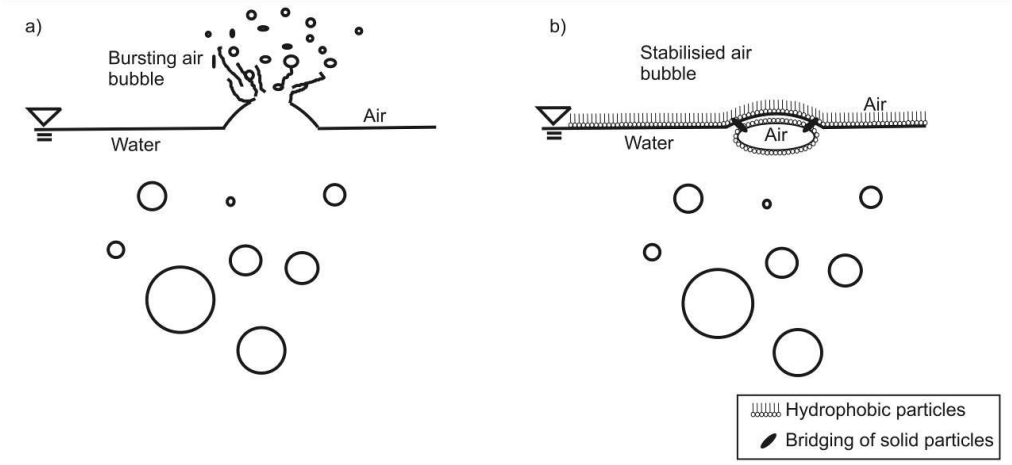


Figure 1 a) the rupture of an air bubble on the water surface **b)** the stabilization of an air bubble due to hydrophobic attachment of foam formers embedded in floc-accumulates. Solid particles can bridge across the air bubble and avert drainage of water which promotes a formation of foam. Adapted from Jenkins et al [13]**.**

Materials and Methods

Sampling Procedure

Waste activated sludge was obtained from West Point Treatment Plant, Seattle, WA and Olympia LOTT Plant, Olympia, WA. Both plants suffered from anaerobic digester foaming. Upon arrival, the sample was stored in four aliquots for light microscopic observation, sludge pretreatment methods, qPCR, and live/dead staining.

Foaming Potential Test

The assessment of the foaming potential was adopted from Ross and Ellis [21]. Briefly, 200 ml of the sludge was transferred into a 1 L graduated cylinder. All tests were conducted in duplicate. The sludge was aerated with a fine bubble diffuser connected with a flow meter to regulate the air flow. The initial height of the sludge was noted and the sludge was aerated at 0.6 L/min for 15 min. The maximum level of foam was recorded to calculate the unstable froth potential (equation 1). After 15 minutes, the air flow

was stopped and the sludge was allowed to settle for a minute. The height of the froth was re-measured after one minute and was used to calculate stable frothing potential (equation 2). The sludge was classified as not foaming if a value of less than 1.0 was measured for unstable foaming potential and a value of less than 0.1 for the stable foaming potential.

$$\text{Unstable Foam Ratio} = \frac{\text{Maximum Froth Height During Aeration (cm)}}{\text{Initial Height of Sludge (cm)}} \quad \text{(equation 1)}$$

$$\text{Stable Foam Ratio} = \frac{\text{Settled Froth Height After 1 Minute Settling Time (cm)}}{\text{Initial Height of Sludge (cm)}} \quad \text{(equation 2)}$$

Live/Dead® BacLight™ Bacterial Viability Kit

Live/Dead® BacLight™ Bacterial Viability Kit was used to differentiate between live and dead cells by microscopy. The kit provides a two-color fluorescence assay for bacterial viability. Syto®9 stains all bacteria whereas propidium iodide penetrates only compromised membranes and causes a reduction in SYTO®9 stain fluorescence when both the stains are present. This kit displays bacteria with intact cell-wall (live) with green and damaged cell walls (dead) with red fluorescence [3].

For this viability test, 0.5mL of each sample was transferred to a clean 1.5mL Eppendorf tube. The samples were centrifuged for 5 minutes at 5,000xg and resuspended in MiliQ water to a final volume of 0.5mL. 1.5 uL of a mixture of SYTO 9 dye (3.34 mM) and Propidium iodide (20 mM) was then added to each sample. Samples were mixed thoroughly and kept in the dark for 15 minutes and examined by microscopically visualization using Axioscop 2 mot plus fluorescence microscope (Carl Zeiss, Mexico).

Pretreatment Methods

Acid/Alkali Pretreatment

The waste activated sludge (WAS) had a solid concentration of 5800 mg/L and was treated with 0.1N HCl and 0.1N NaOH for acidic and alkaline pretreatment respectively. The initial screening of acid/alkali pretreatment was done using WAS from Olympia LOTT Plant. 250 mL of WAS sample was taken and brought to the respective pH value using a pH meter. Prior to the treatment the pH of the untreated sludge was noted. The samples were held for 24h at pH values of 5,5.5,6,9,9.5 and 10. One part of each sample was aerated for 48h and the other was left unaerated (S3). The samples were aerated to activate microorganisms and to hence enhance biodegradability. For the final tests, WAS from West Point Treatment Plant was used. pH values tested in the acidic range were 5, 5.5, and 6, and in the alkaline range were 9,9.5, 10, and 11 and all samples were left unaerated. The holding times for all pH values were varied between 2h, 12h and 24h to observe their effect on foaming index and on the filaments. The samples which were treated at pH 11 were held at room temperature for 2.5h and 24h and additionally at 70^{o}C for 2.5h (S1). Samples at pH 9.5 and 10 were also treated at 70^{o}C for 2.5h. Prior to sampling the batch tests were mixed to ensure a representative sample after anaerobic holding. All samples were tested for their stable (S3) and unstable foaming (S3, Figure 3) potential and were quantitatively estimated for dead cells and *G. amarae* filaments (Figure 4, S2).

Sonication

Sonication is a pretreatment method used to extract the intracellular organic substance of the cell membrane so that it becomes accessible to microorganisms hence increasing the biogas production. The ultrasonic waves cause cavitation, thus rupturing the cell wall enabling microbiological degradation [19]. To investigate the effect of sonication on foam mitigation, the waste activated sludge sample (250 mL) was sonicated in an ultrasonic bath (TI-H-10, Elma ®, Germany) for 30 minutes at 400W and 20kHz and was consecutively tested for the foaming potential.

Acid-Phase Fermentation

Acid-phase fermentation was performed in batch flasks with a 1-day, 2-day, 3-day and 4-day sludge retention time (SRT). The sludge was transferred in four 200 mL Erlenmeyer flasks, which were sealed using rubber stoppers having a gas outlet connected to a manometer. The flasks were then purged with N2 gas to remove oxygen. The samples were mixed once every eight hours. By the end of the experiment the foaming potential was measured. The relationship between decrease in foaming potential and amount of killed filaments was then established using live/dead staining and PMA-qPCR.

Metal Salts

Ferric Chloride ($FeCl_3$) is a commonly used metal salt in wastewater treatment and was used for treating the WAS samples in this study. It was introduced in four different doses of 25, 33, 50, and 260 g/L to the WAS samples (250 mL) and the samples were allowed to settle until coagulation. The foaming potential was then calculated to measure the impact of metal salts in lowering the foaming potential.

Extraction and Quantification of Genomic DNA

PMA treatment and preparation of samples for DNA extraction

PMA (phenanthridium,3-amino-8azido-5-[3-(diethylmethylammonio) propyl]-6-phenyldichloride is a photoreactive dye that binds to DNA. The dye is not able to penetrate the cell membrane of a live cell and hence, does not stain it. However, the cell membrane of a dead cell is compromised, allowing PMA to bind to the DNA of the dead cells causing permanent DNA modifications by covalently reacting with the DNA. The qPCR done for the PMA-stained samples results in a delayed amplification for the dead cells and hence a higher Ct value than it is in case of the live cells. This allows for quantification of the viable cells present in a sample. PMA stained samples are usually exposed to visible light such as a high-power halogen lamp to induce a photoreaction causing PMA to covalently bind to dsDNA.

PMA (Biotium, Hayward, CA) was dissolved in 20% DMSO to obtain a stock solution concentration of 20 mmol/L and aliquots were stored at −20 °C. Since PMA is sensitive to light, the PMA stock solution was prepared under minimal light exposure. For the sample preparation, PMA was added to the cell

samples to get to a final concentration of 200 umol/L and was held for 5 min in the dark with periodic mixing. The sample tubes were then exposed on ice for 4 minutes to a 650 W, 120 V tungsten halogen lamp (B & H PhotoVideo, New York, NY, #GBFAD). After light exposure, samples were transferred to the Lysing Matrix E tube of the FastDNA® Spin Kit for Soil (MP Bio), and DNA was extracted as per protocol instructions. The DNA concentration was measured using NanoDrop spectrophotometer.

Quantitative PCR (qPCR) assays and analyses

The primer and probe assay for total bacterial DNA was designed as previously described [2][22]. Table 1 indicates the 16S rRNA gene primers and probes sets used for *G. amarae* and total bacteria examined in this study. *G. amarae* and total bacteria standard was used as a positive control. Briefly, all reactions used TaqMan® probes and all primers were labeled with 6-FAM as the 5′-terminal reporter dye and TAMRA as a quencher dye at their 3′-terminus. Analyses were performed using an Eppendorf Realplex Mastercycler instrument (Eppendorf). Reagent mixes were prepared by combining 12.5 µl of Ssofast Evagreen Super Mix, 2.5 µl of a mixture of forward and reverse primers (5 µM each), 1 ul of 400 nM TaqMan® probe, and 1.5 µl distilled water per reaction. For each reaction, 20 µl of reagent mix was combined with 5 µl of DNA. The cycling parameters were 30 sec at 95°C for pre-incubation and denaturation of the DNA template, followed by 40 cycles of 95°C for 15 sec for denaturation, 60°C for 120 sec for annealing, and 72°C for 2 minutes for amplification. In every quantitative qPCR run, positive controls were processed as a routine quality control of the assay [15].

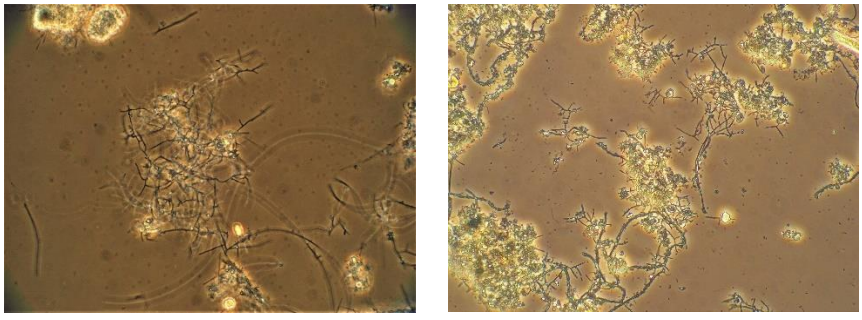
Table 1 Primers and probes set used

Primers	**Target Bacterium**	**Sequence**	**Reference**
G268F	*G. amarae*	CGACCTGAGAGGGTGATCG	[22]
G1096R	*G. amarae*	ATAACCCGCTGGCAATACAG	[22]
1055f	Total bacteria	ATGGCTGTCGTCAGCT	[2]
1392r	Total bacteria	ACGGGCGGTGTGTAC	[2]

Probes			
G-amarae-Probe	*G. amarae*	FAM-ACCTGCTCCTGCATGGGGGTGGG-TAMRA	[2]
16STaq1115	Total bacteria	FAM-CAACGAGCGCAACCC-TAMRA	[11]

Results and Discussion

In this study waste activated sludge obtained from two foaming full-scale wastewater treatment plants was pretreated with acid/alkali treatment, thermal treatment, acid-phase fermentation, sonication, and metal salts to investigate their effectiveness in foam mitigation and killing of the foam forming population. The cause of foaming was found to be primarily *Gordonia amarae* filaments as determined from the microscopic observations (Figure 2) and as confirmed by a positive signal in the qPCR assay (Table 1). Among all the pretreatment methods, acid/alkali pretreatment and acid-phase fermentation proved to be most efficient in killing foam formers and reducing foaming potential while sonication was the least effective. Metal salts gave reasonable results in foam mitigation but were not effective in killing foam formers (Table 2, S2). Therefore, this work focused the most on the foam reduction by acid phase fermentation and acid alkali treatment.



a. b.

Figure 2 Microscopic Image of *G. amarae* Filaments present in

a) Olympia LOTT WAS **b)** West Point WAS**.**

Effect of sonication and metal salts on foaming potential and *G. amarae* bacteria

Upon sonication, it was observed that the WAS started to foam much more than the untreated sludge (50% increase in the unstable foaming index), which might be due to deagglomeration of flocs present in waste activated sludge without killing the bacterial cells [28] (Table 2, S2). The metal salt (FeCl3) was effective in reducing the foaming potential and a maximum decrease (53.5%) was observed with the highest dose of 260 g/L (Table 2). However, the likelihood of metal salts efficiently controlling foaming in anaerobic digesters is questionable as metal salts work on the principle of coagulating the surfactants (including *G. amarae)* such that they flocculate [18] but do not kill the foam-causing bacteria (Table 2, S2). Hence, it is likely that coagulated flocs will disintegrate once the pretreated sludge is fed in the digester and that the foam former will cause frothing issues again.

Effect of Acid/Alkali Treatment on foaming potential and *G. amarae* bacteria

In previous literature, it has been suggested that acid and alkali pretreatment for the waste activated sludge can cause the disruption of the cell membrane releasing intracellular matter into the bulk liquid [5]. To observe the change in the foaming potential upon pH treatment, the samples were treated in a pH range of 5-11. The samples treated at pH 9.5, 10 and 11 were also subjected to a thermal treatment at 70^{o}C, however the thermal treatment was only proven to be effective for pH 11 (S6) which is in line with the results of the PONDUS hydrolysis process which showed good results in increasing biogas production for thermal treatment at pH 11 [27].

All samples (with or without thermal treatment) were measured for their stable (S4) and unstable foaming potential (Table 2). The unstable foaming potential was found to be a much better indicator of the actual foaming conditions than the stable foaming potential because the former takes into consideration the froth

height during aeration which allows the filaments to get attached to the gas bubbles and rise to the surface.

Among various holding times, it was observed that maximum reduction in the unstable foaming potential was obtained in case of the longest holding time of 24h (S5). Graphs (a) and (b) in Figure 3 show the unstable frothing potential values for the pH treatment at a 24h holding time. The results indicated a maximum reduction of 56% in the unstable foaming potential for thermal treatment at pH 11 while the next best reductions of 41.5% and 36.5% were observed in case of non-thermal samples kept at pH 11 and pH 10 respectively for a 24h holding time (Table 2). From the graphs, it can be concluded that the alkaline pretreatment gave equal or better results in lowering the foaming potential as compared to acidic pretreatment when holding time was kept constant. The effect of pH treatment on the foam causing *G. amarae* bacteria was confirmed by live/dead staining (Figure 4, S2) and PMA-qPCR method (Table 2). The microscopic results using live/dead stain showed a greater reduction in live filaments for more alkaline samples as compared to acidic samples. pH 11 thermal treatment was more effective in killing the filamentous bacteria than pH 11 without thermal treatment (Figure 4). pH 10 also showed high concentration of killed cells from the live/dead stained sample (Figure 4).

The qPCR data showed the maximum % kill of 65% for *G. amarae* filaments for pH 11 thermal treatment. Apart from that, pH 11 without thermal treatment with a 24h holding time and pH 10 with a 24h holding time had % kills of 57% and 52%, respectively. Samples that showed a greater reduction in foaming potential also had higher amounts of cells that stained dead from live/dead cell staining (Figure 4) and greater reduction of *G. amarae* in PMA-qPCR (Table 2), suggesting a direct relationship between concentration of *G. amarae* and foaming levels. These results also indicate that pH change either modifies the morphology of the foam causing *G. amarae* or kills them rendering them incapable of attaching to the gas bubbles and rising to the surface. This effect was more pronounced in case of alkaline treatment.

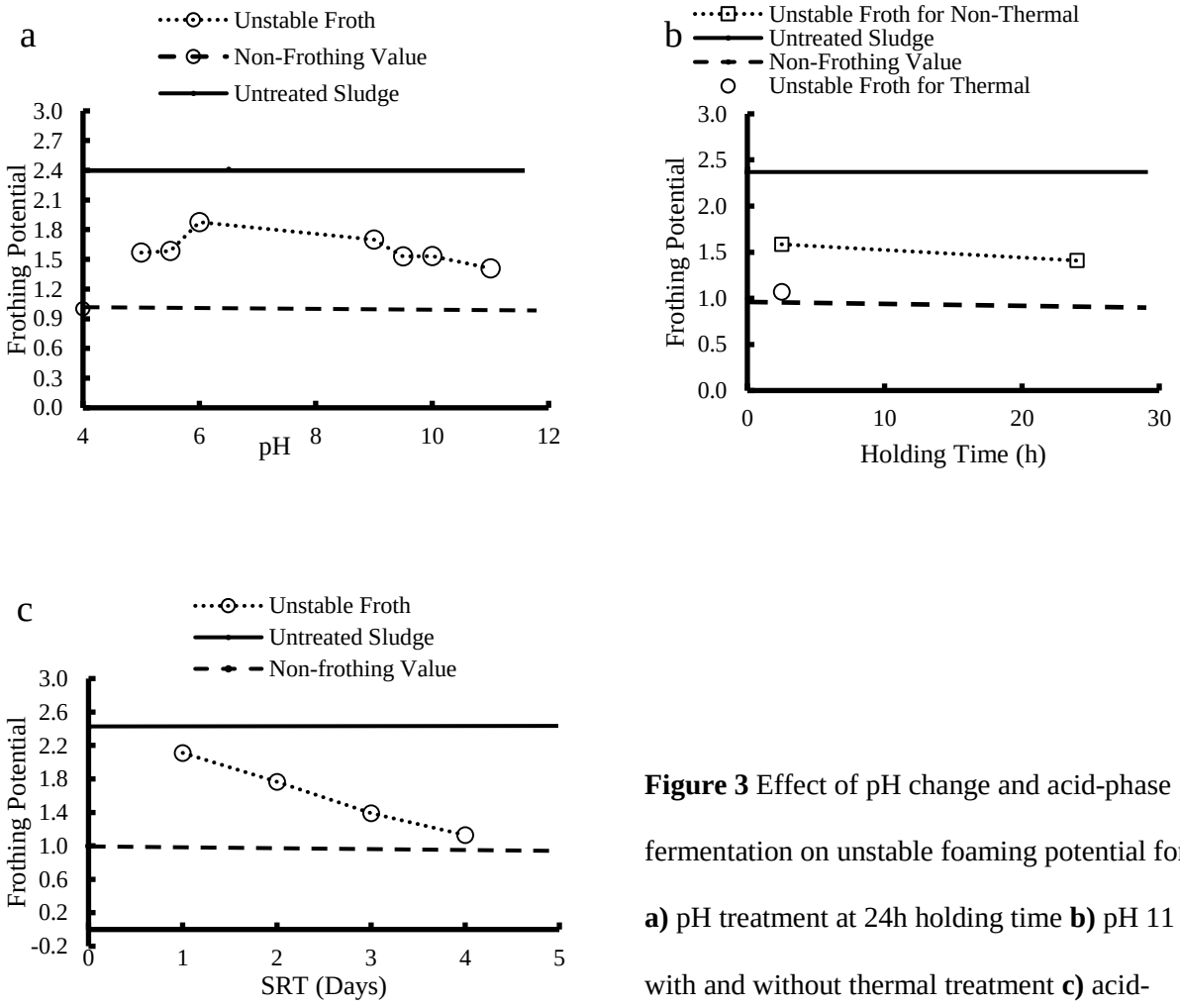


Figure 3 Effect of pH change and acid-phase fermentation on unstable foaming potential for **a)** pH treatment at 24h holding time **b)** pH 11 with and without thermal treatment **c)** acid-phase fermentation batch tests

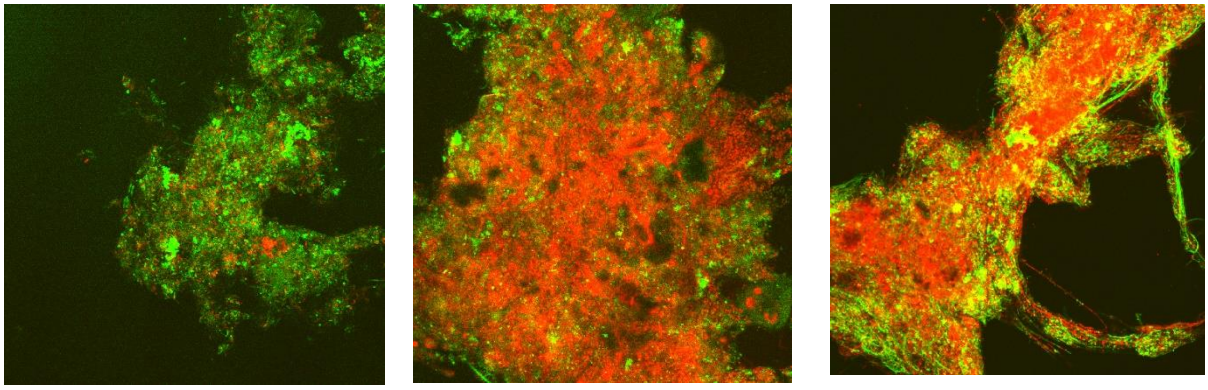
Effect of Acid-Phase Fermentation on foaming potential and *G. amarae* Bacteria

The process of acid phase fermentation separates the acidogenesis and acetogenesis processes from the methanogenesis process, which would elsewise concomitantly occur in a single reactor. In an acid phase fermenter, low HRT is maintained resulting in the accumulation of high amounts of VFAs. This lowers the pH of the fermenter facilitating degradation of proteins and lipids, which in turn increases the methane production due to more substrate availability. In the methane reactor, neutral pH is maintained that promotes the growth of methanogens [9].The effectiveness of acid-phase fermentation in reducing the

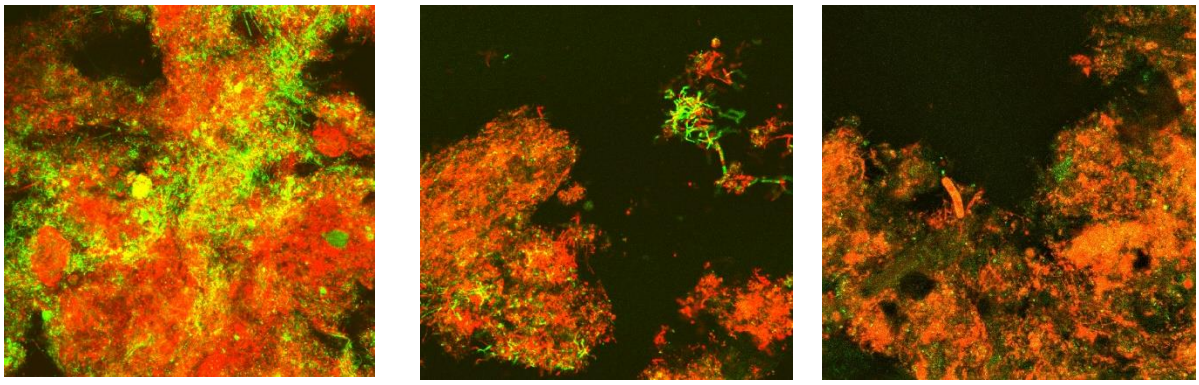
foaming potential can be attributed to a) the killing of foam formers and b) to the chemical effects on the lipids and amino acids leaking out of the cells at lower pH (between 5.6-6 as per this study) [12].

The results showed that a 3-day SRT decreased the pH to 5.71 and 4-day SRT to pH 5.68. The foaming index was decreased by 42% and 53%, respectively (Table 2). This indicates that even though the pH change on going from 3-day to 4-day SRT is insignificant (0.03 pH units), the effectiveness of holding time in foaming potential is considerable (Table 2). The highest concentration of dead *G. amarae* filaments (52%) was observed for a 4-day SRT, which correlated with the highest reduction (53%) in foaming potential (Table 2, Figure 3). The sample with a 1-day SRT was least effective in reducing the foaming potential, which can be explained with a short reaction time of acids on bacterial cells walls leading to a less effective killing and a slightly higher pH value of 5.95 as compared to longer holding times (Table 2, S2). Thus, the holding time played a crucial role in reducing the foaming potential. The live/dead staining (Figure 4, S2) was quantitatively confirmed by PMA-qPCR (Table 2) and the results showed a similar trend in the reduction of live cells along with decreasing foaming potential (Table 2). In summary, longer SRT and higher acidic pH helps in decreasing the foaming potential and killing the foam-forming filaments.

On comparing acid-phase fermentation with acid treatment, it can be observed that with 1-day SRT (pH 5.95), acid-phase fermentation had same % of killed filaments and a comparable reduction in foaming potential as pH 6 with a 24h holding time (Table 2). This indicates that lowering of pH might indeed be the major factor behind the effectiveness of acid-phase fermentation in foam mitigation, without much role of microorganisms.



a. b. c.



d. e. f.

Figure 4 Microscopic images of live/dead stained cells **a)** untreated Sludge **b)** acid-phase 4-day SRT **c)** pH 5.5 at 24h holding time **d)** pH 10 at 24h holding time **e)** pH 11 at 24h holding time without thermal treatment **f)** pH 11 at2.5h holding time with thermal treatment

Table 2% decrease in foaming index and % killed filaments from qPCR

pH/Treatment Condition	**Holding Time (h)**	**% Decrease in Unstable Foaming Index**	**Killed Filaments (%)**
5	2	-10.88	26
5	24	34.95	30

5.5	2	-5.11	35
5.5	24	34.29	39
6	2	-10.17	26
6	24	22.13	39
9	2	-15.40	30
9	24	30	39
9.5	2	-6.85	35
9.5	24	36.53	48
10	2	18.18	43
10	24	36.45	52
5.95	Acid-Phase; 1-Day SRT	12.33	26
5.84	Acid-Phase; 2-Day SRT	26.73	26
5.71	Acid-Phase; 3-Day SRT	42.34	35
5.68	Acid-Phase; 4-Day SRT	53.13	52
11	2.5h; Thermal	56	65
11	2.5h; Non-thermal	34.19	43

11	24h; Non-Thermal	41.51	57
Sonication	0.5	-50	26
Metal Salt ($260g/L\ FeCl_3$)	Until Flocculation	54	13

Conclusions

In this study, various pretreatment methods were studied for their effectiveness in controlling anaerobic digester foaming and reduction in foaming potential was measured before and after pretreatment. The filaments were quantitatively estimated using Live/Dead® BacLight™ Bacterial Viability Kit and PMA-qPCR. The foaming potential results corroborated with the live/dead staining and qPCR results indicating a direct correlation between digester foaming and concentration of the foam forming filamentous *G. amarae*.

Acid / alkali treatment and acid phase fermentation both showed a high reduction in foaming and in inactivation of filaments and an additional thermal treatment enhanced the effectiveness of foam reduction. Sonication showed no effect on foaming and on *G.amarae* filaments whereas addition of $FeCl_3$ although did decrease the foaming potential, did not kill bacterial cells.

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