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Enhanced anaerobic co-digestion of fat, oil, and grease by calcium addition: Boost of biomethane production and microbial community shift

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ABSTRACT

This work focused on the application of calcium (0.1–1% w/v) to overcome the inhibition caused by the high loadings (2% v/v) of fat, oil, and grease (FOG) in the context of biomethane production, organic removal, and microbial community shift. Addition of 0.5% calcium showed maximum biomethane production (6-fold increase); biomethane production decreased following the addition of calcium (> 0.5%). The highest organic removal rates were 83 and 89% upon the addition of 0.3 and 0.5% calcium, respectively. Addition of calcium facilitated the growth of bacteria of phylum *Firmicutes* from the *Clostridium*, *Syntrophomonas,* and *Sedimentibacter* genera. The population of members from the genus *Methanosaeta* increased after the addition of 0.5% calcium, which is one of the factors responsible for high biomethane production. This study demonstrated that addition of calcium is an attractive strategy to avoid the inhibition of the growth of anaerobic microflora due to the presence of high FOG concentrations.

1. Introduction

Anaerobic digestion (AD) is one of the most attractive approaches for treatment of organic wastes owing to its beneficial characteristic such as significant decrease in sludge biomass, relatively low energy consumption, and generation of renewable energy in the form of biogas (Ziels et al., 2018). All types of organic wastes can be used as substrates for AD, including food waste, municipal sludge, organic portion of household waste, farm waste, and commercial solid waste (Tyagi et al., 2018). About 40–50% of the organics present in sludge are converted to methane, resulting in low digestion effectiveness and biomethane production (Park et al., 2016). Classic digester product recovery systems can recover 20–40% of the energy used for the operation of wastewater treatment plants (WWTPs) (Habashi et al., 2016; Long et al., 2012).

Anaerobic co-digestion (ACD) of organic wastes, including palm oil mill effluents, corn silage, rice straw, and fat, oil, and grease (FOG), with sludge provides an economic opportunity for the development of ACD proficiency and consequent biomethane production (Salama et al., 2019). FOG exits in municipal sewage, edible oils, slaughterhouses, and food wastes (Rasit et al., 2015). Addition of FOG as a co-substrate during the AD of animal manure or wastewater sludge to enhance methane production has gained attention because of the high biomethane potential (1.0 m^3 CH_4/kg volatile solids) of FOG (Hosseini and Wahid, 2013; Jeganathan et al., 2006). Biomethane generation from FOG can be inhibited by limited microbial activity, which is caused by the toxicity of long-chain fatty acids (LCFAs) at high concentrations.

Lipidic-wastes containing fatty acids with more than seven carbon (> C7) chains have been reported to result in the complete inhibition of microbes during AD (Long et al., 2012; Roy et al., 1985; Wang et al., 2018). FOG is converted to glycerol and LCFAs that naturally have 14–24 carbon atoms (Tezel et al., 2008). Under anaerobic environment, LCFAs are converted via ß-oxidation to lower molecular structures such as acetate. Low solubility and adsorption of LCFAs, and their growth-inhibitory actions towards anaerobes have been reported as the cause of the various operational issues in the anaerobic digestion treatment of wastes with excessive lipid content (Amha et al., 2017). LCFAs inhibit methanogens; this is primarily attributed to the perpetual toxicity due to cell damage, and is known to affect both syntrophic acetogens and methanogens. Accumulation of LCFAs has been hypothesized to harm cell membranes, lower nutrients transport, and limit cell permeability, thereby disturbing the cell's capability to control the pH (Palatsi et al., 2009). Increase in LCFA concentrations has been reported to prolong

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the lag phase of digestion. Several studies have reported the permanent inhibition of the growth of methanogens due to high levels of synthetic LCFAs (Angelidaki and Ahring, 1992; Hanaki et al., 1981; Rinzema et al., 1994).

Studies on the use of calcium or bentonite in AD have mostly focused on oil mill effluents, swine wastewater, and synthetic LCFA-based substrates to decease the toxicity of single or multiple LCFAs to the microbes involved in the AD (Ahn et al., 2006; Beccari et al., 2001; Kumar et al., 2016). Different loadings of FOG (0.2, 0.8, and 1.8% v/v) were applied in AD, and biomethane production was increased with FOG addition. However, it was observed that the biomethane production was inhibited at the 1.8% (v/v) FOG loading (Martinez et al., 2011). Studies regarding the use of calcium to lower the inhibition of FOG containing multiple LCFAs during ACD are rare, and it is interesting to evaluate the effects of calcium during the co-digestion of FOG. In the current study, various calcium concentrations were applied to explore the optimum calcium level that can decrease the toxicity of 2% FOG and boost biomethane production. The positive and negative effects of calcium on the ACD of FOG were observed in the context of biomethane yield and COD elimination. Microbial community analysis was also performed to identify the microbial community after the addition of calcium.

2. Materials and methods

2.1. Feedstock sampling

Primary sludge, waste-activated sludge, and anaerobic digestion sludge were sampled from a local domestic wastewater treatment plant in Daegu, South Korea. FOG was obtained from the Resource Recycling Center, Songsan-myeon, Dangjin-si/Ji-Gun E&M, South Korea. All samples were rapidly transported to the laboratory after collection and were kept at 4 °C.

2.2. Experimental design for anaerobic co-digestion

Experiments were carried out in 500 mL bottles with a working volume of 60% to evaluate the impact of varying calcium concentrations (0.1, 0.3, 0.5, 0.7, and 1% w/v) with high loadings of FOG (2% v/v) on ACD. Calcium hydroxide ($Ca(OH)_2$, Japan) and calcium chloride ($CaCl_2{\cdot}2H_2O$, Merck) were used at a ratio of 1:1 to attain the desired calcium concentration in each reactor. FOG was mixed with calcium before the addition of the sludge to decease the toxic effects of LCFAs on the anaerobic microflora (Fig. 1). The bottles were then seeded with 48% of the sludge mixture (primary sludge and waste-activated sludge) and 50% of anaerobic digestion sludge. The pH of the reactors was maintained at ~7 by HCl. N_2 gas was used for flushing the bottles followed by closing the bottles tightly with rubber stoppers. The bottles were incubated at 150 rpm at 37 °C.

2.3. Analytical procedures

2.3.1. Feedstock characterization

The nitrogen (N), carbon (C), and phosphate (P) contents of the sludge were investigated as previously described (BIS: 9234-1979; Kumar et al., 2016). The total solids (TS) and volatile solids (VS) were analyzed by the gravimetric method. Metal ions were evaluated by an ELAN DRC II ICP-MS system (PerkinElmer Sciex, USA). The pH of the solution was assessed using an Orion 5-Star pH/ORP/Conductivity/DO Meter (Thermo Scientific, USA). Physicochemical characterization of the substrates showed that the pH of the anaerobic digestion sludge was neutral, which is a distinctive quality of wastewater. The TS contents in the anaerobic digestion sludge were comparatively lower than those of the primary sludge and waste-activated sludge, because of the thickening of the sludge at the WWTPs. FOG contains fatty acids that results in acidic pH and a high lipid content (94%). The high contents of lipids in FOG resulted in high carbon content (76.75%) and hydrogen (13.29%), thus making it a very high carbonaceous waste with C/N ratio (1 1 9). Metal ions such as iron and zinc were the most dominant, among the other metal ion species, in the FOG (Table 1).

2.3.2. Gas and liquid phase analysis

Biogas was collected in Tedlar bags and the volume of gas was quantified manually (Ahn et al., 2006). The biomethane was analyzed using the Agilent GC 7890B system (Palo Alto, CA, USA), which has an HP-PLOT/Q column (30 m × 0.32 mm × 20 μm) and a thermal conductivity detector (TCD). The temperatures were set at 120 and 150 °C for the inlet and detector, respectively. The oven temperature was fixed at 45 °C (Kurade et al., 2019). A modified Gompertz equation was useful in describing the methane production curves in the batch kinetic assays (Saha et al., 2018), which is as follows:

$$M = M_{max}\exp\left\{-\exp\left(\frac{R_{max} \times e}{M_{max}}(\lambda - t) + 1\right)\right\} \quad (1)$$

M (mL) is the cumulative methane yield, M_{max} (mL) is the total amount of methane produced in time t, R_m (mL/d) is the maximum methane production rate, λ (d) is the lag phase, t is the incubation period (d), and e is 2.718.

Five-milliliter aliquots were collected at regular time intervals for COD analysis. The samples were filtered with 0.2 μm syringe filters (Sartorius), and the supernatant was used for the COD analysis. COD was measured as previously described (Method 5220D; closed reflux, colorimetric method; Eaton et al., 2005).

2.4. Microbial diversity analysis

2.4.1. Isolation of DNA and 16S rRNA sequencing

High-throughput sequencing of 16S rRNA amplicons was used to evaluate the diversity of the microbial communities present at the various calcium concentrations (Kleinsteuber, 2018). Samples were collected from the control reactors and the 0.1, 0.5, and 1% calcium-containing reactors for DNA extraction using the QIAamp DNA tool kit (Qiagen, Valencia, CA, USA). DNA confirmation and quantification were performed using agarose gel electrophoresis (1.2 wt%), and the picogreen technique (Invitrogen) using Victor3 fluorometry. The DNA collected from each sample was about 50–100 ng, and it was kept at –20 °C for further investigation. For amplification of V3–V4 variable regions in the 16S rRNA gene, a previously established protocol was used (Saha et al., 2019a). The multiplexing was implemented using the Nextera XT Index Kit (Illumina) after 16S amplification. To confirm the size, 1 μL of each PCR product was evaluated via Bio-analyzer DNA 1000 chip. A 2 × 300 bp paired-end run (MiSeq reagent kit v3) was performed on an Illumina MiSeq platform to sequence the libraries.

2.4.2. Bioinformatics data analysis

PEAR software was used to combine the Miseq paired-end reads, and Trimmomatic v0.35 was used for the quality trimming of the demultiplexed amplicon read pairs (Bolger et al., 2014; Zhang et al., 2014); next, the quality control of the raw data was performed to filter out reads with quality scores < 30 using FastQC v0.11.4 (Andrews, 2010). The QIIME software package was used to analyze the clean reads (Caporaso et al., 2010). Resemblance of 97% was chosen for operational taxonomic units (OTUs) alongside sequences from the Greengenes 16S rRNA database, and coordinated with recognized bacterial genomes to classify the members of the hypoxial community. Comparative abundance was calculated based on the OTUs. The MetaCoMET web platform was used to calculate the relative abundance among each group (temporal).

2.4.3. Metagenome sequencing and statistical analysis

The α-diversity values were evaluated by using the Simpson

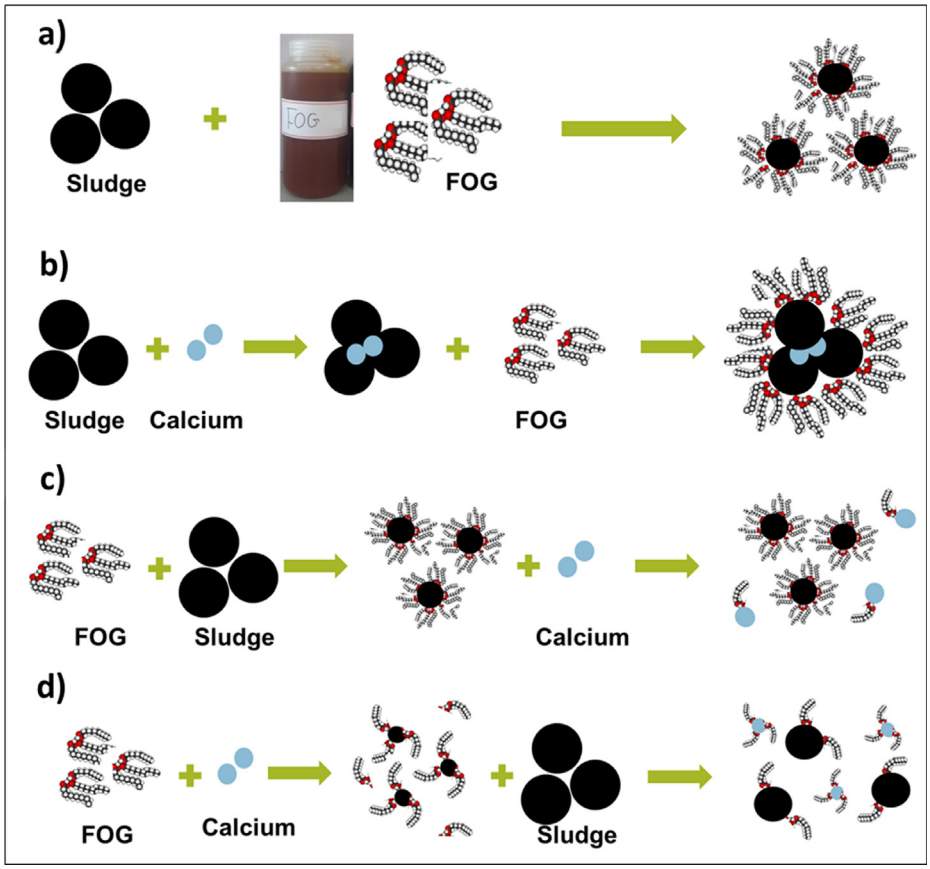

Fig. 1. Various approaches for calcium addition during the anaerobic co-digestion process: (a) Performing the FOG co-digestion without calcium addition resulted in FOG-mediated inhibition of the growth of microbes. As the microbes were coated with FOG, cellular transportation became limited, which affected substrate access and subsequent biogas release, (b) Calcium addition before FOG: Calcium aggregated with the inoculum, and later FOG covered these aggregates, which also caused the inhibition of microbial growth, (c) Calcium addition after FOG: As the inoculum is already covered with FOG, calcium provided no help to the microbes, and (d) The best approach was to mix the calcium with FOG before the inoculum addition, which reduced the growth-inhibition effects of FOG.

Table 1
Physicochemical characteristics of the seed sludge and substrates used in this study.

Parameters	FOG	PS + WAS	ADS
pH	4.56	5.90	6.94
Total solids (wt.%)	95.40	3.48 ± 0.01	3.39 ± 0.03
Volatile solids (wt.%)	96.80	77.41 ± 0.18	70.03 ± 0.004
Ash (wt.%)	3.05	0.79 ± 0.003	1.02 ± 0.002
Fixed carbon (wt.%)	0.15	23.38 ± 0.001	30.99 ± 0.18
Total lipid (wt.%)	94.0	–	–
Total protein (wt.%)	4.00	–	–
Total carbon (wt.%)	76.75	–	–
Total nitrogen (wt.%)	0.64	–	–
Total hydrogen (wt.%)	13.29	–	–
C/N ratio	119.92	–	–
Calorific value (MJ/kg)	14.15	18.70 ± 0.0002	20.19 ± 0.03
Calcium (mg/L)	23.09	41 ± 2.99	64.5 ± 3.19
Magnesium (mg/L)	0.11	9 ± 1.45	54.6 ± 2.97
Aluminum (mg/L)	0.40	0.06 ± 0.01	1.5 ± 0.01
Cupper (mg/L)	1.57	0.01 ± 0.0	0.4 ± 0.01
Cobalt (mg/L)	0.17	–	–
Iron (mg/L)	56.38	0.14 ± 0.02	2.0 ± 0.03
Zinc (mg/L)	42.31	–	–

FOG = Fat, oil, and grease.
PS = Primary sludge (60%).
WAS = Waste active sludge (40%).
ADS = Anaerobic digestion sludge.

diversity indexes and OTU numbers, with phylogenetic distance as the markers. The relationship between microbial communities in the reactors and the performance of the reactors were determined by calculating the β-values of the microbial communities, which were analyzed using principal component analysis (PCA) with the STAMP software package. The data of triplicate experiments in this work are presented as the mean and standard deviation values. Statistical analyses were performed according to our previous report (Kurade et al., 2019).

3. Results and discussion

3.1. FOG composition and the role of calcium in lowering FOG-mediated inhibition of the growth of microbes

FOG contained medium-chain fatty acids (C9–C12) and LCFAs (> C12), which accounted for 1.2 and 98% of FOG, respectively, and have growth-inhibitory effects. The primary LCFAs present were palmitic (18%), oleic (33%), and linolenic acids (17%) (Table 2). Kobayashi et al. (2014) showed similar results to those observed in this study, and reported that FOG extracted from grease trap waste also contained mainly oleic (30.7–41.1%), linoleic (22.8–35.8%), and palmitic acids (15.2–19.0%). Growth inhibition by LCFAs during ACD depends on the microbial population and the type of LCFAs. LCFAs such as palmitic, oleic, linoleic, and stearic acids in FOG have been recognized for their high growth-inhibitory effects (Pereira et al., 2005) because of the adsorption of the LCFAs onto the cell wall, which affects membrane transport (Dasa et al., 2016). This adsorption delays biomethane production; however, it can be prevented by introducing a competitive synthetic adsorbent.

To minimize the toxicity of LCFAs to the microbes involved in AD, calcium and bentonite were applied (Ahn et al., 2006; Beccari et al., 2001; Kumar et al., 2016). However, reports on the use of calcium to reduce the toxic effects of FOG containing multiple LCFAs (Table 2) are rare. The main mechanisms for the addition of calcium during the process of AD are shown in Fig. 1. In the absence of calcium, FOG covers the sludge (microbial inoculum), thereby blocking the cellular transport in bacteria, and inhibiting their growth (Fig. 1a). Addition of calcium to the AD sludge, followed by FOG addition (Fig. 1b), is not suggested, as the calcium aggregates with the sludge particles that are covered with FOG. On the contrary, the addition of FOG to AD sludge,

Table 2
Fatty acid composition of fat, oil, and grease (FOG) including short chain fatty acids, medium chain fatty acids, and long chain fatty acids.

Fatty acid methyl esters (FAMEs)	Value (%)
Caproic acid methyl ester (C6:0)	0.4
Caprylic acid methyl ester (C8:0)	0.1
Capric acid methyl ester (C10:0)	0.1
Undecanoic acid methyl ester (C11:0)	0.3
Lauric acid methyl ester (C12:0)	0.4
Tridecanoic acid methyl ester (C13:0)	0.5
Myristic acid methyl ester (C14:0)	1.8
Myristoleic acid methyl ester (C14:1)	0.1
Pentadecanoic methyl ester (C15:0)	0.2
Palmitic acid methyl ester (C16:0)	18.2
Palmitoleic acid methyl ester (C16:1)	0.2
Heptadecanoic methyl ester (C17:0)	0.3
Cis-10-Heptadecenoic methyl ester (C17:1)	0.3
Stearic acid methyl ester (C18:0)	6.8
Oleic acid methyl ester (C18:1n9t)	33.4
Linolelaidic acid methyl ester (C18:2n6t)	14.0
Linoleic acid methyl ester (C18:2n6c)	0.7
γ-Linolenic acid methyl ester (C18:3n6)	17.6
Arachidic acid methyl ester (C20:0)	0.1
Linolenic acid methyl ester (C18:3n3)	2.0
Cis-11-Eicosenoic acid methyl ester (C20:1)	0.3
Heneicosanoic acid methyl ester (C21:0)	0.0
Cis-11 14-Eicosadienoic acid methyl ester (C20:2)	1.0
Cis-8 14 17-Eicosatrienoic acid methyl ester (C20:3n6)	0.1
Behenic acid methyl ester (C22:0)	1.10
Saturated fatty acids (SFAs)	30.20
Unsaturated fatty acids (USFAs)	69.70
Short chain fatty acids (SCFAs)	0.50
Medium chain fatty acids (MCFAs)	1.20
Long chain fatty acids (LCFAs)	98.2

SCFAs ($< C_8$).
MCFAs (C_9-C_{11}).
LCFAs ($> C_{13}$).

followed by calcium addition, is also not a preferable method, because FOG can still cover/coat the microbes and inhibit their growth (Fig. 1c). This study proposed that the most appropriate approach for calcium addition was to mix the calcium with FOG before the addition of AD sludge (Fig. 1d), which resulted in the reduction of the growth-inhibitory effects of FOG at the beginning of the digestion (Ma et al., 2015; Pereira et al., 2005).

3.2. Effect of calcium on biomethane production and organic removal during AD

The successful operation of AD is indicated by organic removal and the conversion of the organics to biomethane. The relationship between different calcium additions and biomethane production is given in Fig. 2. Addition of 2% (v/v) FOG during the co-digestion without calcium supplement resulted in the inhibition of biomethane production, which can be attributed to the presence of LCFAs. A regression analysis of the experimental sets showed that the cumulative methane production was well fitted with the modified Gompertz model Eq. (1), as it showed an R^2 value between 0.98 and 0.99 (Fig. 2). Martinez et al. (2011) also reported that biomethane was inhibited after 1.8% (v/v) FOG loading. Several studies have reported the inhibition of methanogens such as *Methanospirillum hungatei*, *Methanospirillum formicicum*, *Methanosaeta concilii*, and *Methanosarcina mazei*, due to LCFAs (Silva et al., 2016; Sousa et al., 2013). This inhibition is because of the coating of microbes with the LCFAs, which limits cellular transportation, hinders substrate access, and subsequently decreases biogas production (Long et al., 2012; Ma et al., 2015). Addition of 0.1% calcium to the reactors enhanced the biomethane production, compared to that of the control. Increase in the calcium concentration to 0.3% enhanced methane production by 5-fold. However, a significant increase in methane

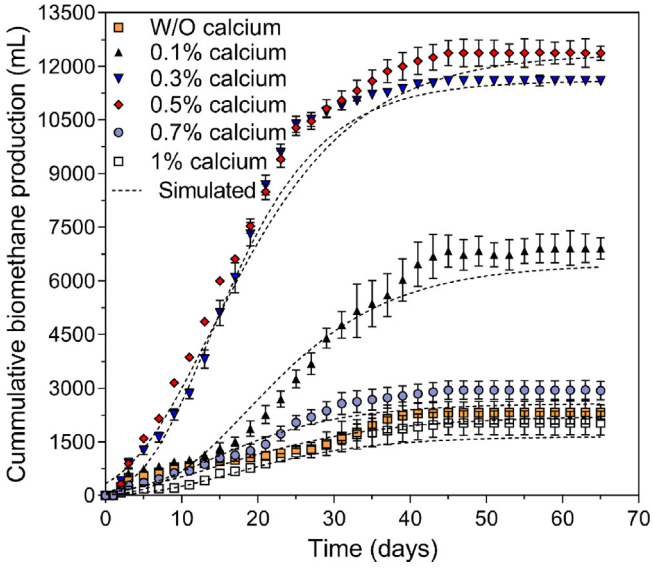

Fig. 2. Improvement in methane production during the anaerobic co-digestion of FOG (2% v/v) in the presence of various concentrations of calcium.

production (6-fold) was observed following the addition of 0.5% calcium (Fig. 2). Reports on anaerobic granular sludge have shown the suppression threshold of calcium to be 0.5% in both static reactor and laboratory-scale expanded granular sludge bed reactor (Dang et al., 2014). Various ions such as calcium ions have been identified to be essential as cofactors for polysaccharide production. Calcium ions can also enhance the permeability of the cellular outer membranes of Gram-negative bacteria, mostly the species involved in acetogenesis (Huang and Pinder, 1995). At high calcium concentrations of 0.7–1%, the methane production was inhibited, and it was observed to be less than that of the control following the addition of 1% calcium (Fig. 2). It has been reported that calcium has a growth-inhibitory effect at concentrations higher than 0.5% (Kumar et al., 2016). This inhibition is because the extreme quantity of calcium results in the precipitation of phosphate and carbonate, causing the scaling of biomass, lowering the activity of methanogens, and the loss of buffering ability and vital nutrients in the AD process (Huang and Pinder, 1995; Kumar et al., 2016). High calcium concentrations affect the bio-treatment productivity because of the precipitation of calcium in the granular sludge, which reduces the microbial activity (Liu et al., 2011).

Organic removal was also affected by the addition of calcium to the AD reactors. COD was reduced from 131 to 14–64 g/L after the addition of 0.1–1% calcium. The highest COD removal efficiencies were 83 and 89% at 0.3 and 0.5% calcium concentrations (Fig. 3), respectively, which resulted in the highest biomethane production (Fig. 2). At higher concentrations of calcium (0.7–1%), COD reduction efficiency decreased and even became lower than that in the control following the addition of 1% calcium; COD reduction rate and biomethane production decreased under these high calcium concentrations. The operation of an AD system without lipids/LCFAs may not require the addition of calcium (Kumar et al., 2016; Luo et al., 2015). However, if the AD process is performed in the presence of lipids/LCFAs, addition of calcium is required to decease the toxicity of LCFAs (Ma et al., 2015). Increase in the lipid/LCFA concentrations during AD may require the addition of increased concentrations of calcium. Ahn et al. (2006) reported that during an AD process where the lipid concentration was 20%, the addition of 0.3% calcium resulted in 67% COD removal and the production of 32 L of biomethane. In another study, biomethane production of up to 13 kg COD/m^3 was observed upon the addition of 0.7% of calcium in an AD process which had a lipid content of 37.2% (Ma et al., 2015).

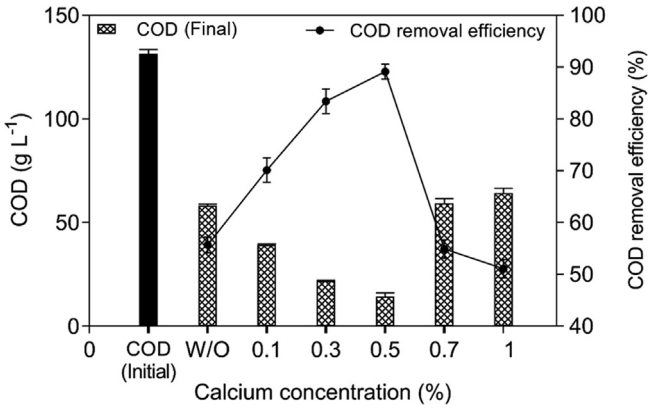

Fig. 3. Effects of different calcium concentrations on organic removal during the anaerobic co-digestion of FOG.

3.3. Microbial community shift upon calcium addition during AD

Evaluation of microbial communities following the addition of various concentrations of calcium during AD with co-substrates such as FOG has not yet been explored well. The microbial community change in the absence of calcium and following the addition of a single concentration of calcium has been studied previously (Luo et al., 2015); however, the relationship between calcium content and microbial community shift is still unclear. Data of 16S rRNA amplicons from high-throughput sequencing have shown that calcium addition altered microbial community during AD in the presence of FOG. The dissemination of OTUs in the Venn diagram was made to recognize the variations in the overall and taxa-specific OTUs upon calcium addition (Fig. 4a). Among all the reactors, the samples from those containing 0.5 and 1% of calcium shared the maximum number of OTUs, accounting for 11.7% and 10.2% of their own OTUs, respectively. Only 380 OTUs were found in all samples. As the calcium concentration increased the number of common OTUs reduced, thus indicating a very high diversity among the control samples and samples from reactors containing 1% calcium (only 3.3% of OTUs were common OTUs). The lowest number of unique OTU counts was observed in samples from the control reactors (without calcium). The diversity of microbial communities was suppressed in the presence of only FOG. The microbial diversity was comparatively higher in the reactors containing 1% calcium, which showed 938 OTUs associated with other reactors, in which the OTU counts were low; this may be due to the increase in the populations of other microbes. The principal component analysis (PCA) indicated that calcium addition had a notable impact on the microbial communities. Microbial communities were not clustered in the PCA plot, and a larger separation of microbial populations was observed, indicating a distinct diversity of the microbial communities in all four reactors, and that the microbial communities are sensitive to the calcium concentration (Fig. 4b). *Firmicutes* (19–40%) and *Bacteroidetes* (17–25%) were the major phyla in all the reactors (Fig. 5) given their dominant role in hydrolysis and hydrogenogenic acidogenesis (Adam and Perner, 2018; Saha et al., 2019a). Adding calcium amplified the relative abundance of members from the phylum *Firmicutes*, which are recognized to produce proteases, cellulases, lipases, and other extracellular enzymes, and are closely interrelated with the degradation of organics and acid formation (Luo et al., 2015).

The abundance of *Bacteroidetes* decreased following the addition of 0.1 and 1% calcium, possibly due to the FOG-mediated toxicity and inhibition of their growth; however, it was almost unchanged when the calcium concentration was 0.5%, which was optimum for the maximum conversion of FOG to biomethane (Figs. 2 and 5). The microbial communities within the phylum *Bacteroidetes* are known to produce different enzymes, including lyases, hydrolases, lipases, and ligases, which can degrade complex organic compounds and synthesize acetate (Chen et al., 2007). The abundance of *Spirochaetes* and *Chloroflexi* increased to 4% and 10% at calcium concentrations of 0.1 and 0.5%, respectively; however, their abundance decreased at a calcium concentration of 1%. Members from the phylum *Chloroflexi* have been reported to be sensitive to higher concentrations of calcium, and have been found to be present in low abundance in the presence of high calcium concentrations, leading to low COD removal (Dang et al., 2014). Addition of calcium facilitated an increase in the population of the acidogenic genera *Clostridium, Syntrophomonas*, and *Sedimentibacter* in the phylum *Firmicutes*. The abundance of *Clostridium* was suppressed at a calcium concentration of 0.5%, and increased effectively at high calcium concentrations; however, at low calcium concentrations (0.1%), members from the genus *Syntrophomonas* almost doubled in abundance, but significantly decreased at higher calcium concentrations. Dang et al. (2014) have also reported that at a calcium concentration of 0.5% during AD, members from the genus *Clostridium* survived the calcium supplements and were more abundant than other microbes. Syntrophic bacteria are reported to be sensitive to LCFAs (Ma et al., 2015), and are influenced by the addition of calcium (Lorowitz et al., 1989). Unlike *Clostridium* and *Syntrophomonas*, in this work, the richness of members

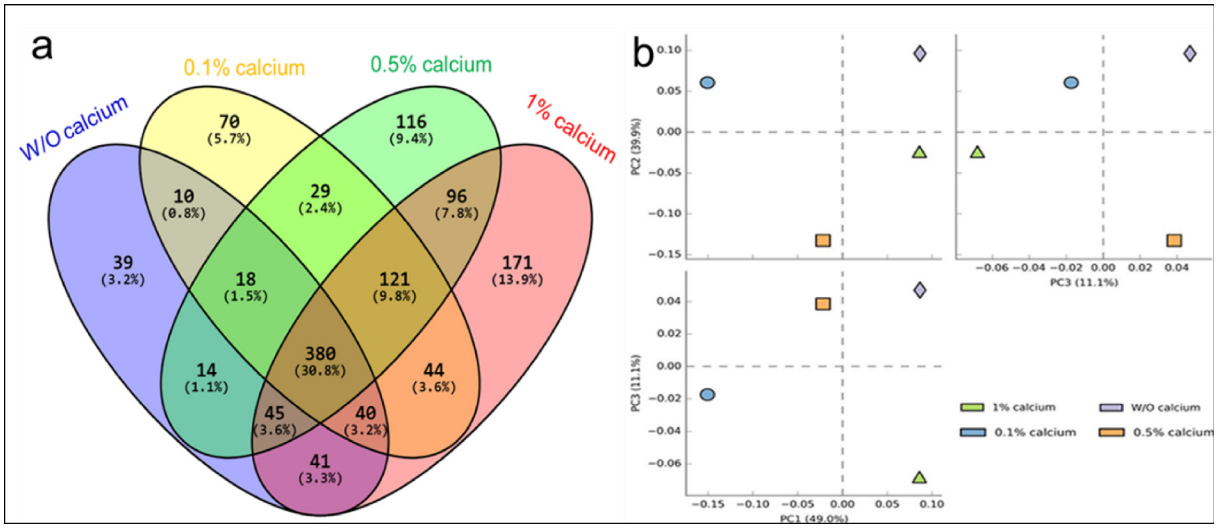

Fig. 4. Distribution of operational taxonomic units (OTUs) (A), Shannon indices (B), and the principal component analysis (PCA) of the bacterial genera identified in the different reactors (C) in the presence of different concentrations of calcium.

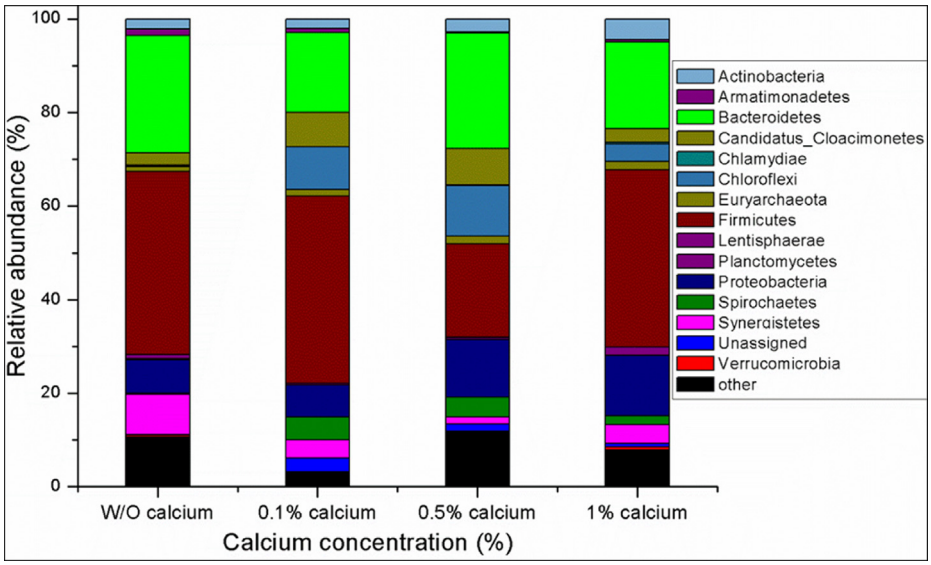

Fig. 5. Microbial community changes and the relative abundance of the phyla after exposure to various calcium concentrations. Microbial communities were evaluated after the completion of the digestion process in the control reactors and the reactors containing 0.1%, 0.5%, and 1% calcium using the high-throughput sequencing of 16S rRNA amplicons.

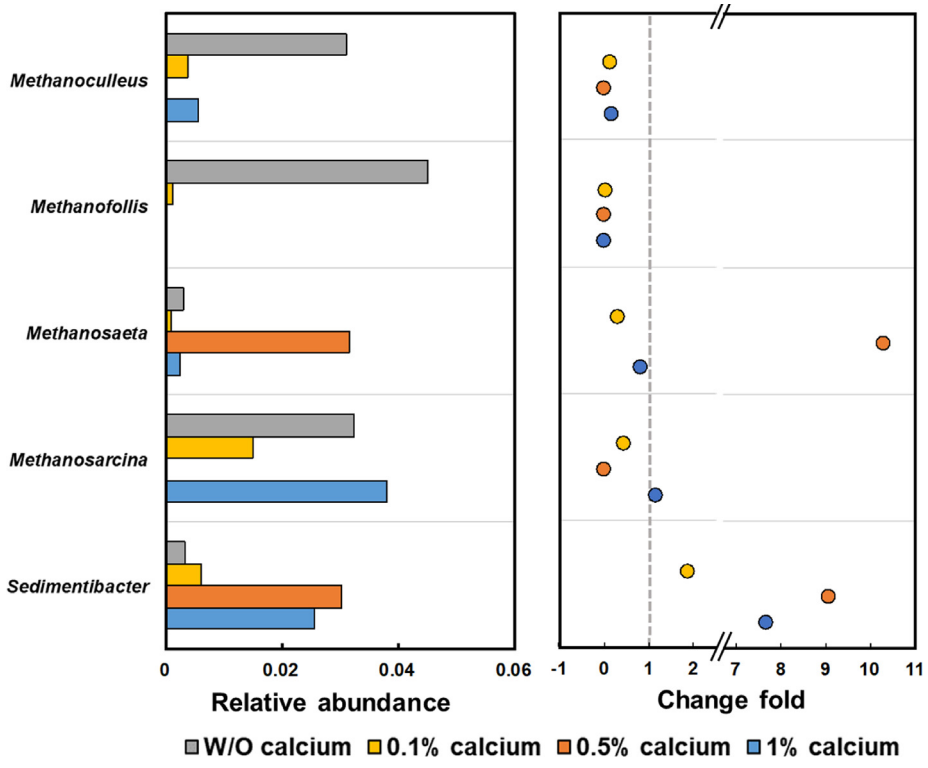

Fig. 6. Effect of calcium addition on changes in the relative abundances of Methanogens and Syntrophic bacteria.

from the genus *Sedimentibacter* increased with increase in calcium levels and was the highest at a calcium concentration of 0.5%, indicating the role of the members from this genus in FOG degradation at optimum calcium concentrations; thus, members from this genus facilitate high methane production (Fig. 6). *Sedimentibacter* and *Syntrophomonas* are reported to be actively involved in acetogenesis and the transfer of electrons to methanogens, resulting in increased biomethane production (Fitamo et al., 2017; Saha et al., 2019b).

A negative shift in the abundance of members from the phylum *Firmicutes* showed a negative shift in the reactors containing 0.5% calcium, and remained unchanged at calcium concentrations of 0.1 and 1% (Fig. 5), representing their dynamic contribution in the degradation and consumption of FOG, and acidogenic H_2 generation; this demonstrates their ability to withstand the growth-inhibitory effects of FOG at low and high levels of calcium. Luo et al. (2015) have performed an AD process without FOG addition, and observed a high abundance of members from the phylum *Firmicutes* in the presence of calcium. Wan et al. (2016) have reported the increased abundance of members from *Firmicutes* in their mesophilic acidogenic reactors. At an optimum calcium concentration (0.5%), members from the bacterial phyla *Bacteroidetes* (24.57%), *Firmicutes* (19.95), *Proteobacteria* (12.35%), *Chloroflexi* (10.90%), and *Candidatus-Cloacimonetes* (7.78%) were abundant, and among archaeal communities, members from the phylum *Euryarchaeota* (1.6%) were dominant, and high biomethane production was observed (Fig. 2), which demonstrates the active role of these bacteria in the successful conversion of FOG to biomethane. The abundance of members from the other phyla either remained unchanged or decreased upon calcium addition. Bacteria from the phyla

Firmicutes and *Bacteroidetes* have been stated as active contributors in FOG degradation (Sousa et al., 2007). Methanogens nominated as *Euryarchaeota* were present in the reactors from all the experimental groups. Among the methanogenic genera, *Methanosaeta* and *Methanosarcina* (in phylum *Euryarchaeota*) were influenced by calcium addition, and the abundance of the genus *Methanosaeta* significantly increased (ca.10-folds) along with *Sedimentibacter* (ca.9-folds) upon the addition of 0.5% calcium, indicating their active role in FOG biomethanation (Fig. 6). Dang et al. (2014) have observed increase in the population of the genus *Methanosaeta* following calcium addition during AD. Survival of *Methanosaeta* in FOG-containing reactors has also been attributed to their acetoclastic and FOG-tolerant nature (Carr et al., 2017; Singh et al., 2016; Ziels et al., 2016).

4. Conclusions

High loadings of fat, oil, and grease (FOG) inhibit the growth of key microorganisms in anaerobic digestion (AD). Optimum calcium concentration (0.5%) showed maximum COD removal and methane production, and was associated with the dominance of the phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Chloroflexi*, and *Euryarchaeota*, which are actively involved in the conversion of FOG/LCFAs to biomethane. Addition of higher calcium concentrations (0.7–1%) was not preferable in the digester as inhibition of methane production was observed. This study concluded that addition of calcium may be necessary to avoid the growth-inhibitory and toxic effects of high loadings of FOG in AD systems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.122353.

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