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Microbial community acclimatization for enhancement in the methane productivity of anaerobic co-digestion of fats, oil, and grease

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ABSTRACT

The methane productivity and long chain fatty acids (LCFAs) degradation capability of unacclimatized seed sludge (USS) and acclimatized seed sludge (ASS) at different substrate ratios of fats oil and grease (FOG) and mixed sewage sludge were investigated in this study. Biogas produced in ASS in initial phase of anaerobic digestion had higher methane content (65–76%) than that in USS (26–73%). The degradation of major LCFAs in the ASS was 22–80%, 33–191%, and 7–64% higher for the substrate ratios of 100:10, 100:20, and 100:30, respectively, as compared to the LCFAs' degradation in USS. Microbial acclimatization increased the population of Firmicutes (40%), Bacteroidetes (32%), Synergistetes (10%), and Euryarchaeota (8%) in ASS, which supported the faster rate of LCFAs degradation for its later conversion to methane. The significant abundance of *Syntrophomonas* and *Methanosarcina* genera in ASS supported faster generation rate of methane in an obligatory syntrophic relationship.

1. Introduction

Anaerobic co-digestion (ACoD) is gaining a lot of scientific attention recently because of its capability of enhanced production of methane, improved degradation/utilization of substrates, and sustainable approach of solving environmental issues (Wang et al., 2013; Kurade et al., 2019). It involves a combination of two or more substrates, such as lignocellulosic waste, food waste, fats, oil, and grease (FOG) along with wastewater sludge (Li et al., 2013; Salama et al., 2019). Despite its high productivity, utilization of a high strength organic substrate, such as FOG, face numerous technical challenges in a co-digestion approach. The deleterious effects of accumulation of long-chain fatty acids (LCFAs) in a digester, which can create severe problems in overall methane generation process, has been studied extensively (Palatsi et al., 2009; Silva et al., 2016). The accumulation of LCFAs limit cell permeability and mass transport, damage the cell membranes, and inhibit acetogens and methanogens at high concentrations of FOG.

The major bacteria involved in anaerobic digestion (AD) are severely affected by the presence of high concentrations of LCFAs in the reactor; however, the inhibition caused by LCFAs is a reversible process which can be neutralized. Inhibited microorganisms including syntrophic acetogens and methanogens, regain their original metabolic activities once the LCFA-biomass associated degradation had recommenced (Palatsi et al., 2009). The AD inhibition caused by LCFAs is one of the most serious problems in biogas plants; thus, several studies were conducted to develop methods to overcome such inhibitions and to facilitate a stable operation for co-digestion. Angelidaki and Ahring (1992) studied addition of adsorbents to minimize the inhibitory effects of LCFAs. The incorporation of easily digestible co-substrates, such as glucose and cysteine, were useful in overcoming LCFAs inhibition (Kuang et al., 2006). Discontinuous feeding of the substrate to the AD for promoting the development of active anaerobic community, which can effectively degrade the lipids has also been suggested (Cavaleiro et al., 2008; Nadais et al., 2006).

The acidification of the reactor is also a common problem during codigestion of high strength organics such as lipids and food waste (Zeng et al., 2019). This causes the imbalance in the microbial communities which affect the initial AD processes making system failures and instability. Thus, acclimation of AD inoculum prior to the substrate addition is required in order to counteract this problem. A few studies in the past have demonstrated the effectiveness of microbial acclimation step in AD to several substrates/compounds such as food waste, cheese whey and vegetables fruit waste, humic acid, phenols and ammonia which minimized the negative impact of inhibitory compounds and achieved greater substrate digestion and gas productivity (Wilson et al., 2013; Madigou et al., 2016; Chang et al., 2018; Li et al., 2019).

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Recently, an acclimated culture of propionate degrading methanogens exhibited increased tolerance to acidic environment with stable methane production between pH 4.8 and 5.5 (Li et al., 2018). Chang et al. (2018) also observed accelerated process rate of hydrogenogenic acidogenesis and carboxylic chain elongation due to utilization of an acclimatized microbiome, which yielded 31% higher specific hydrogen production potential compared to unacclimatized microbiome during the fermentation of mixed fruit waste.

Acclimatization of the anaerobic process to LCFAs has been reported earlier. Continuous or pulse exposure has increased tolerance to LCFAs and improved bioenergy recovery (Alves et al., 2001; Cavaleiro et al., 2008; Palatsi et al., 2009). The improved methane productivity through acclimated microbial communities is due to the consumption of substrate without lag time, and the produced intermediary substrates do not accumulate in the digester (King et al., 2011). An anaerobic microflora acclimatized with gradual dosing of FOG showed a significant improvement in hydrogen yield (72%) with volatile solid reduction up to 65%, compared to unacclimatized culture (Saha et al., 2019a). The authors concluded that, complete degradation of major unsaturated fatty acids in the highest FOG loading was due to the preference of the acclimatized microbiome. Enhancing the resistance of anaerobic processes through microbial acclimation to high dosage of substrate can provide a suitable method to improve the methane generation capability of microorganism without facing any process inhibition (Zeng et al., 2019). Some microorganisms cannot withstand the stress caused by the addition of a new, high strength organic substrate, while others acclimatize to the new conditions, and the substrate shows an increased dominance in the anaerobic system. Therefore, generally, microbial acclimation involves significant changes in the microbial communities present in the sludge.

Recently, we provided an insight into fundamental mechanism that regulates the methanogenic activities under the inhibitory conditions caused by the shock dosing of FOG into the digester (Kurade et al., 2019). In line with our previous demonstration, the current study is an extensive investigation to explore differential capability of acclimatized and unacclimatized seed sludge. The primary objective of this study is to investigate the differential capability of acclimated and unacclimatized seed sludges. The methane productivity and LCFAs degradation capability of unacclimatized seed sludge (USS) and acclimatized seed sludge (ASS), along with their microbial dynamics were investigated with different total solids substrate ratios. High throughput Illumina MiSeq sequencing of 16S rRNA amplicons was implemented to reveal the dynamics of bacterial and archaeal communities of USS and ASS to explore the differential mechanisms of USS and ASS. The outcome of this study can be useful to implement the improvised strategies for better substrate digestibility and greater methane production.

2. Materials and methods

2.1. Collection of wastewater sludge and lipidic substrate

The wastewater sludge, which included primary, activated, and anaerobically digested sludge (ADS), were collected from the Jungnang sewage treatment plant, Seoul, Korea in 20 L polyethylene cans. The FOG waste was obtained from the Resource Recycling Center, Dangjin, Republic of Korea. All samples were transferred to the laboratory and quickly stored at 4 °C after its collection until further use.

2.2. Development of highly enriched FOG consuming microbial consortium

The anaerobic microbial consortium was developed in 1 L airtight anaerobic co-digesters made up of glass material in fed-batch mode. Predigested ADS was used as the seed inoculum. The co-digesters were fed 400 mL of seed inoculum, 75 mL of mixed thickened sludge (mixture of primary sludge (PS) and waste activated sludge (WAS) at a ratio of 70:30, v/v), and 25 mL of FOG. The volatile solids VS of FOG added to the digester in the first batch was 72% of the total volatile solids of the anaerobic co-digester. L-cysteine HCl (0.5 g L^{-1}) and sodium bicarbonate (5 g L^{-1}) were added to the digester as a reducing agent and pH buffer, respectively. The co-digesters were flushed with ultrapure N_2 gas (99.99%) and then closed with airtight plastic-rubber caps to maintain anaerobic conditions. After gas production reached the saturation point, the digesters were again fed 10 mL of FOG (2% v/v). This fed-batch process was continued for ten cycles, and the recovered contents of the co-digester were further used as highly enriched FOG using microbial consortium. The consortium development was conducted in a shaking incubator (150 rpm) at 37 °C (mesophilic methane production).

2.3. Batch anaerobic co-digestion with unacclimatized and acclimatized microbial consortia

Airtight glass anaerobic co-digesters (500 mL capacity) were used for the methanogenic batch with working volume of 400 mL. The collected ADS without any further changes was the unacclimatized seed inoculum, recovered from Jungnang sewage treatment plant, Seoul, Korea. This ADS was never exposed to lipid rich substrate. The acclimatized microbial consortium was developed in the laboratory through the fed-batch operation described in Subsection 2.2. Further, 360 mL of both acclimatized and unacclimatized predigested seed inocula were transferred to separate co-digester bottles (the seed sludge was void of biogas production because of exhaustion of substrates). Mixed sludge (MS; mixture of PS and WAS at a ratio of 70:30, v/v) and FOG were added as co-substrates to these co-digester bottles in three different proportions (details presented in Table 1). L-cysteine HCl (0.5 g L^{-1}) and sodium bicarbonate (5 g L^{-1}) were added to the digester as a reducing agent and pH buffer, respectively. The co-digesters were flushed with ultrapure N_2 gas (99.99%) and then closed with airtight plastic-rubber caps to maintain anaerobic conditions. All treatments were performed in triplicate and were kept in a shaking incubator (150 rpm) at 37 °C throughout the experiment.

2.4. Analytical characterization

2.4.1. Properties of wastewater sludge and FOG

The proximate and ultimate compositions of sludge and substrates, and their physical properties were determined according to the protocols reported earlier (Saha et al., 2018). The colorimetric phenol–sulfuric acid method was used to estimate the total carbohydrate

Table 1
The total solids and organic loadings of the digesters with unacclimatized and acclimatized seed sludge.

	Substrate ratio (MS:FOG) (%TS)	Total solids loading of the co-digester (g L^{-1})				
		Seed sludge	MS	FOG	TS of digester	VS of digester
Unacclimatized seed sludge (USS)	100:0 (Control)	22.32	3.74	0	26.06	17.17
	100:10	22.32	3.72	0.36	26.08	17.50
	100:20	22.32	3.71	0.74	26.10	17.85
	100:30	22.32	3.69	1.09	26.12	18.18
Acclimatized seed sludge (ASS)	100:0 (Control)	20.79	3.74	0	24.53	14.65
	100:10	20.79	3.72	0.36	24.55	14.98
	100:20	20.79	3.71	0.74	24.57	15.33
	100:30	20.79	3.69	1.09	24.59	15.66

MS: Mixed sludge (Mixture of thickened primary and waste activated sludge (70:30)).
FOG: Fats, oil, and grease.
TS: Total solids.
VS: Volatile solid.

content of the sludge and substrates. Total nitrogen (wt%) was used as a function for determining the total protein content of all the sludge and substrates. Total lipid was estimated using the chloroform–methanol extraction method.

2.4.2. Headspace methane gas determination

Biogas was recovered from the headspace of the co-digesters into gas bags at regular time intervals. The volume of the gas was measured using a 60 mL Luer syringe, and the head space of the digesters was also considered to calculate the total volume of gas produced. The biogas composition was analyzed using gas chromatography (GC, 7890B Agilent Technologies, Palo Alto, CA, USA). The GC was equipped with HP-PLOT/Q column (30 m × 0.32 mm × 20 μm) and a thermal conductivity detector (TCD). The inlet, column, and TCD temperatures were maintained at 120, 45, and 150 °C, respectively, under a constant pressure of 10.232 psi. Argon was used as the carrier gas. A 100 μL gas sample was injected into the GC column using an airtight sample lock syringe (Hamilton, PA, USA). A standard calibration curve of a gas mixture [CH_4 (39.72%, mol/mol), H_2 (25.06%, mol/mol), and CO_2 (24.78%, mol/mol) balanced in N_2 gas (purity-99.99%) (Greengas, Seoul)] was used for determining the methane content in the retrieved biogas from the co-digesters. 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:

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

M (mL) is the cumulative methane yield, M_{max} (mL) is the total amount of methane produced in time t, R_m (mL d^{-1}) is the maximum methane production rate, λ (d) is the lag phase, t is the incubation period (d), and e is 2.718.

2.5. Determination of microbial community structure

2.5.1. Isolation of DNA and high-throughput sequencing of 16S rRNA

The samples were collected, their DNA was isolated using QIAamp DNA Stool Kit (Qiagen, Valencia, CA, USA), and they were further subjected for metagenomic analysis of 16S rRNA amplicons to reveal population density of the microbial communities. The V3-V4 variable region of the 16S rRNA gene was targeted for screening of bacterial biodiversity. The bacterial and archaeal primers were designed according to the recent reports Wuchter et al. (2013) and Ziels et al. (2016), and the details are presented in Table 2. Library quantification was performed by real-time PCR using a CFX96 real-time system (BioRad, Hercules, CA, USA), and the libraries were sequenced using a 2X300-bp paired-end run (MiSeq Reagent Kit v3) on an Illumina MiSeq platform.

2.5.2. High-throughput sequencing data analysis

The PEAR software was used to join the MiSeq paired-end reads, and Trimmomatic v0.35 was used for trimming the demultiplexed amplicon read pairs (Bolger et al., 2014). The raw data was processed by FastQC v0.11.4 to filter out the reads with quality scores below 30. The retrieved clean reads were evaluated using the open-source QIIME software package (Caporaso et al., 2010). Operational taxonomic units (OTUs) corresponding to each read were chosen at 97% similarity against the Greengenes 16S rRNA database and matched with the known bacterial genomes to identify members of the hypoxial community. The OTUs were used to quantify the relative abundance. A comparative analysis was performed using the MetaCoMET web platform to determine the relative abundance between each group.

2.6. Statistical analysis

The data were presented as the mean and standard deviation of the triplicate experiments. Statistical analysis was performed using the IBM SPSS software version 21.0 for Windows. One-way analysis of variance using the Tukey–Kramer multiple comparison was used to analyze the difference between the treatments. The variations were considered statistically significant at a confidence interval of $p < 0.05$.

3. Results and discussion

3.1. Physico-chemical properties of seed sludge and substrates

The characteristics of different sludge and substrates used in this study are presented in Table 3. Both seed sludges (unacclimatized and acclimatized seed sludge) and MS had nearly neutral pH, which is a normal characteristic of municipal wastewater treatment plant's (WWTP's) sludge. The FOG waste had acidic pH because of the presence of fatty acids. The major fatty acids in the FOG were oleic acid (24%), palmitic acid (13%), γ-linolenic acid (13%), linolelaidic acid (10%) and stearic acid (5%) as the dominant LCFAs. The co-digestion substrates contained higher solids as compared to the seed sludge because of the thickening process of the WWTP. The ASS contained relatively lower volatile solids than the USS because of excessive digestion of the co-digestion substrates during the acclimation fed-batch process. The carbohydrates and protein fractions mainly contributed to the organic contents in the sludge. The presence of high amount of proteins in the sludge resulted in their low C/N ratio. In contrast, the high amounts of triglycerides in the FOG waste made it a carbonaceous substrate with an extremely high C/N ratio. The C/N ratio is an important factor for establishing a successful anaerobic digester, with an optimum range of 20–30 (Kurade et al., 2019). It controls ammonification, acidification of digesters caused by β-oxidation of fatty acids, and effective utilization of substrates (Oh and Martin, 2010; Saha et al., 2019a). The low C/N ratio of the sludge was compensated by the high C/N ratio of FOG, which prevents rapid acidification of digester during the hydrolysis of fatty acids in FOG and maintains an ideal pH for acidogenic fermentation.

3.2. Methane productivity of unacclimatized and acclimatized seed sludges

The methane productivities of both unacclimatized and acclimatized seed sludges were observed for various FOG loadings. The USS showed 10, 27, and 49 mL of ultimate methane production (g^{-1} VS_{added}) with 100:10, 100:20, and 100:30 substrate ratios, respectively (Fig. 1a). The methane production in the ASS was significantly higher than their respective counterparts of USS. It exhibited 31-, 34-, and 17-fold greater methane generation than that in USS with 100:10, 100:20, and 100:30 substrate ratios, respectively. The statistical analysis confirmed the considerable ($p < 0.001$) increase in the generation of

Table 2
The primers used for the amplification of bacterial and archaeal microbial RNA.

SN	PCR region	Primer name and sequence	Reference
1.	Bacterial 16S PCR amplification	519F (5′-CCTACGGGNGGCWGCAG-3′) 806R (5′-GACTACHVGGGTATCTAATCC-3′)	Wuchter et al. (2013)
2.	Archaeal sequence library	Arch-349F (5′-GYGCASCAGKCGMGAAW-3′) Arch-915R (5′-GTGCTCCCCCGCCAATTCCT-3′)	Ziels et al. (2016)

Table 3
Physico-chemical properties of different sludges including anaerobically digested seed sludge and MS, FOG. The values are the mean and standard deviation (SD) of triplicate analyses.

		Seed inoculum		Co-digestion substrate	
Properties		USS	ASS	MS	FOG
Physical properties	pH	6.92 ± 0.02	7.14 ± 0.01	6.52 ± 0.03	4.74 ± 0.02
Proximate analysis	TS (g L^{-1})	24.8 ± 0.22	23.1 ± 0.62	37.4 ± 0.24	951 ± 12.3
	VS (g L^{-1})	16.1 ± 0.15	13.3 ± 0.11	26.8 ± 0.15	909 ± 2.23
	VS/TS (wt%)	65.1 ± 0.69	57.5 ± 0.19	71.6 ± 0.60	95.6 ± 0.62
	Ash (wt%)	1.23 ± 0.11	0.69 ± 0.01	0.95 ± 0.03	3.62 ± 0.09
	Fixed carbon (wt%)	38.47 ± 2.14	32.2 ± 0.57	30.2 ± 0.12	0.21 ± 0.02
	Total carbohydrate (wt%)	7.14 ± 1.02	4.41 ± 0.24	15.4 ± 0.04	ND
	Total protein (wt%)	38.5 ± 1.2	34.6 ± 0.41	35.4 ± 1.02	8.25 ± 0.28
	Total lipid (wt%)	ND	ND	6.04 ± 0.08	93.56 ± 3.24
Ultimate analysis	Total carbon (wt%)	35.4 ± 0.15	29.2 ± 0.14	40.2 ± 0.61	76.58 ± 1.14
	Total nitrogen (wt%)	5.21 ± 0.05	4.28 ± 0.02	4.89 ± 0.05	1.04 ± 0.07
	Total hydrogen (wt%)	6.14 ± 0.012	5.02 ± 0.01	6.14 ± 0.01	14.25 ± 0.24
	Total sulfur (wt%)	0.82 ± 0.02	0.63 ± 0.05	0.95 ± 0.05	1.58 ± 0.08
	Total oxygen (wt%)	52.43 ± 0.35	60.87 ± 0.14	47.82 ± 1.13	6.55 ± 0.59
	C/N ratio	6.79	6.82	8.22	73.6

USS: Unacclimatized seed sludge.
ASS: Acclimatized seed sludge.
MS: Mixed sludge (Mixture of thickened primary and waste activated sludge (70:30)).
FOG: Fats, oil, and grease.
TS: Total solids, VS: Volatile solids.

methane owing to FOG addition. A regression analysis of the experimental data exhibited that the cumulative methane production was well fitted with the modified Gompertz model with the R^2 values between 0.89 and 0.99 (Table 4). The methanogenic activity of ASS was exceedingly higher at all the studied substrate concentrations (4 to 20-fold) than that of USS, which resulted in maximum utilization of substrates within 12 days of digestion. Among all the tested digesters, ASS with 100:30 substrate ratio exhibited maximum methane production rate (Rm- 6.86 mL d^{-1}) and greater methanogenic activity (1.43 mL g^{-1} $VS_{initial}$ d^{-1}). Moreover, the ASS did not experience any inhibitory effects of FOG loadings because of continuous adaptation of ADS microflora to FOG which resulted in faster hydrolysis and utilization of the substrate as compared to USS. The methane production in ASS on the 12th day of codigestion was 38-, 44-, and 36- fold higher as compared to that in USS, which shows that the USS exhibited slower production of methane in all the studied substrate ratios because of exposure to the new substrate (FOG). The ASS digesters utilized almost all the available substrates during first 12 days and reached their saturation point where methane production was halted/slower. At the same time, the USS digesters were facing their exponential phase where maximum methane production was observed.

The ratio of methane to carbon dioxide (CH_4/CO_2) at the beginning of AD (1 d) was very low (42–48% methane) in almost all the digesters, including USS and ASS (Fig. 1b). As digestion progressed, the CH_4/CO_2 ratio in the ASS (in all three sets) significantly improved as compared to that in the USS. The highest CH_4/CO_2 ratio in ASS (100:10 to 100:30) was observed between 4th and 12th day of digestion with 65 to 76% methane in the biogas; whereas, it was relatively lower in the ASS (within the range of 26–73%). However, this trend was reversed after 12 days, when the maximum conversion of substrates in the ASS occurred with a high methane production rate, and the earlier inhibition of LCFAs in the USS was neutralized by its microbial communities. This indicates that the CH_4/CO_2 ratio is dependent upon the methanogenic activity with a positive correlation.

The co-digestion of FOG is usually associated with numerous problems, which include accumulation of LCFAs, sludge foaming, decrease in pH caused by the accumulation of volatile organic acids (Wan et al., 2011; Li et al., 2013). Such issues ultimately deteriorate the optimum conditions of AD and decreases digestion efficiency and methane generation. The LCFAs in FOG caused rapid acidification in digestion broth; however, the addition of pH buffer and high buffering capability of the sludge maintained the pH above neutral level, with exception in USS with 100:30 substrate ratio, wherein pH dropped below 7 on the 8th day (Fig. 2). The excessive dosage of FOG (100:30) and the unexposed microflora of USS to FOG, which could not maintain the pace between volatile fatty acid formation and methane production reactions, were the major reasons of pH decrease in USS at high concentration of FOG. The hydrolysis of FOG into LCFAs and glycerol was performed instantaneously during the initial phase of ACoD. The LCFAs were further converted to methane via intermediate production of acetate through syntrophic contributions of β-oxidizing bacteria and methanogenic archaea. However, this conversion step was the most rate-limiting process in ACoD (Cirne et al., 2007). Thus, degradation of LCFAs being comparatively slower than hydrolysis of lipids caused over-accumulation of LCFAs in the digesters. The accumulated LCFAs adsorbed into the surface of methanogenic bacteria and caused deteriorated mass transfer effects and inefficient substrate utilization, which eventually inhibited methanogenesis (Silva et al., 2016; Ziels et al., 2017, 2018). Moreover, the delayed methane production in USS can be clarified by the fact that, USS was never exposed to highly carbonaceous substrates, such as FOG. Thus, it required a buffer time to withstand the high loads of LCFAs generated during lipid hydrolysis. Contrastingly, the ASS was sequentially fed with FOG for several batch cycles and adapted to the excessive loading of the lipidic substrate. Therefore, it showed seamless production of methane even with high concentration of FOG (100:30) in the digesters (Fig. 1a). These results indicated that the acclimated microflora in the ASS utilized maximum amount of substrate with enhanced production of methane within a short duration, unlike USS with the extended lag phase. In an earlier investigation, adaptation of fermentative microbiota to xylan-rich medium enhanced methane production by 53% from hemicellulosic feedstock (Weiss et al., 2010). An increased hydrogen productivity by 3.5 times was observed during the fermentation of thin stillage due to acclimatization of microbiota to glucose (Nasr et al., 2011).

3.3. Degradation of LCFAs in USS and ASS

LCFA profiling of two sets of digesters (USS and ASS) elucidated the

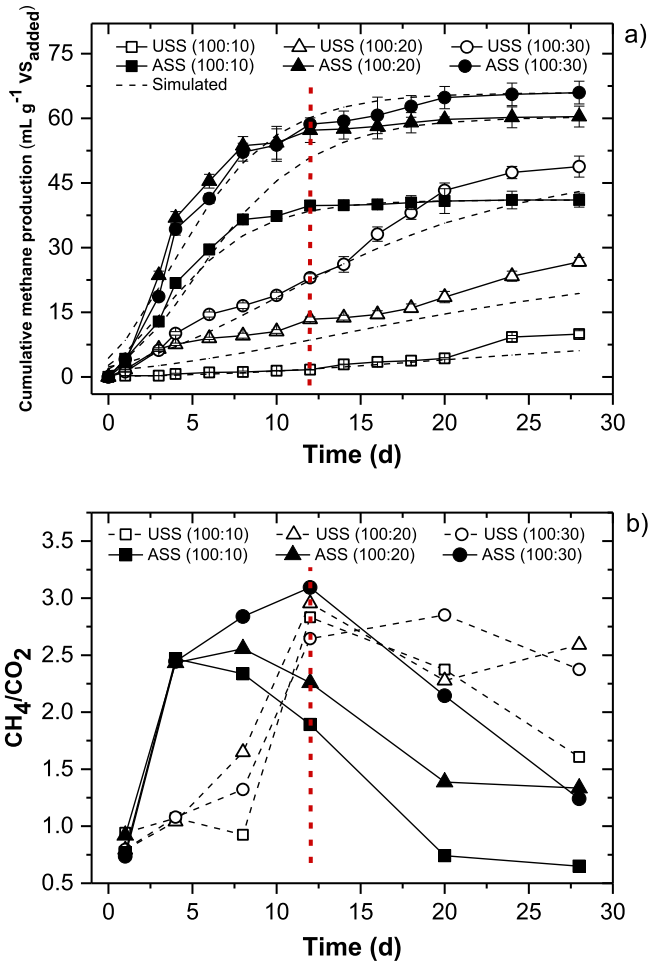

Fig. 1. Cumulative methane production (a), and comparison between the methane content of biogas produced (b) during co-digestion of FOG in the digesters containing unacclimatized seed sludge (USS) and acclimatized seed sludge (ASS) at different substrate mixing ratios of mixed sludge to fats, oils and grease (MS:FOG) under mesophilic conditions. The vertical red line indicates the end of the exponential phase in the ASS digesters. The error bars represent the standard deviation of the mean (n = 4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fate of LCAFs during anaerobic co-digestion. The major LCFAs, such as myristic, palmitic, stearic, and oleic acids, in the USS degraded in the range of 26–51%, 17–70%, and 28–51% for the substrate ratios of

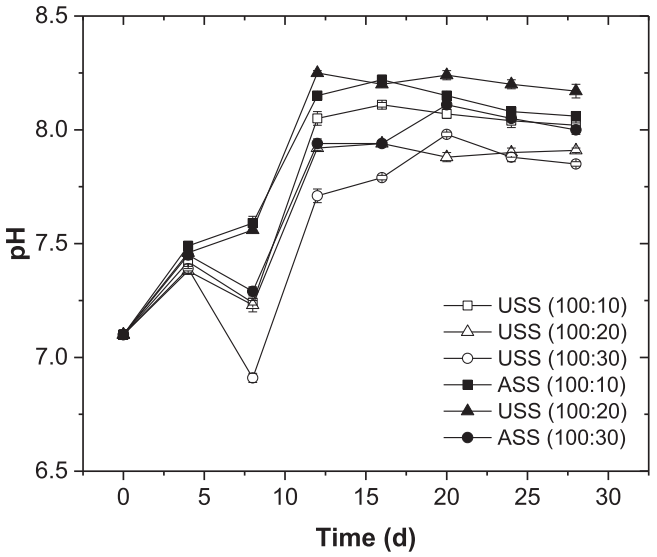

Fig. 2. The changes in pH of the digesters containing unacclimatized seed sludge (USS) and acclimatized seed sludge (ASS) at different substrate mixing ratios of mixed sludge to fats, oils and grease (MS:FOG).

100:10, 100:20, and 100:30, respectively (Fig. 3). The degradation of these LCFAs in the ASS was significantly higher than that in the USS. It was observed that, stearic acid was highly degraded in the studied digesters, with an average degradation of 57 and 83% in the USS and ASS (at all three-substrate ratios), respectively. In contrast, oleic acid was resistant to biodegradation because it showed an average degradation of 32 and 44% in USS and ASS, respectively. The FOG used in this study comprised oleic acid (24%), palmitic acid (13%), and stearic acid (5%) as the prevailing LCFAs (data not presented). It has been reported that the toxicity of these acids was relatively higher as compared with others LCFAs with IC_{50} in the order of oleic acid (75 mg L^{-1}) > palmitic acid (1100 mg L^{-1}) > stearic acid (1500 mg L^{-1}) (Palatsi et al., 2009). Other research investigations also provided the inhibitory levels of these LCFAs with large variations, 30–880 mg L^{-1} for oleic acid, 30–200 mg L^{-1} for linoleic acid, and 1100–1500 mg L^{-1} for palmitic acid (Nakasaki et al., 2019). The existence of excessive amounts of these LCFAs in the FOG could have imposed high toxicity on the microbial cells, which ultimately caused inhibition in methane generation in the USS at an earlier phase of anaerobic co-digestion. However, the microbial adaptation in ASS was useful in overriding the inhibitory effects of these LCFAs, thereby resulting in seamless production of methane without showing any signs of inhibition of LCFAs. Faster degradation of LCFAs in anaerobic reactor due to microbial acclimation was in line with the observations of Silva et al. (2014), who reported

Table 4
Regression analysis of the methane production data between the experimental and modeling values obtained using the Gompertz equation, maximum methane production rates and methanogenic activities of the different sets.

	Substrate ratio in USS digester			Substrate ratio in ASS digester		
	100:10	100:20	100:30	100:10	100:20	100:30
R^2	0.93	0.94	0.99	0.99	0.89	0.98
Standard error	0.86	1.78	2.15	1.70	7.45	3.14
F-value	161	218	767	953	93.07	684
Significance F	2.51×10^{-8}	4.7×10^{-9}	3.04×10^{-12}	8.4×10^{-13}	5.27×10^{-7}	5.96×10^{-12}
p-value	0.19	0.096	0.32	0.40	0.03	0.47
Rm	0.28	0.78	2.04	4.72	5.59	6.86
MA	0.049	0.13	0.35	0.99	1.17	1.43

USS: Unacclimatized seed sludge.
ASS: Acclimatized seed sludge.
Rm: Maximum methane production rate (mL d^{-1}).
MA: Methanogenic activity (1.43 mL g^{-1} $VS_{initial}$ d^{-1}).

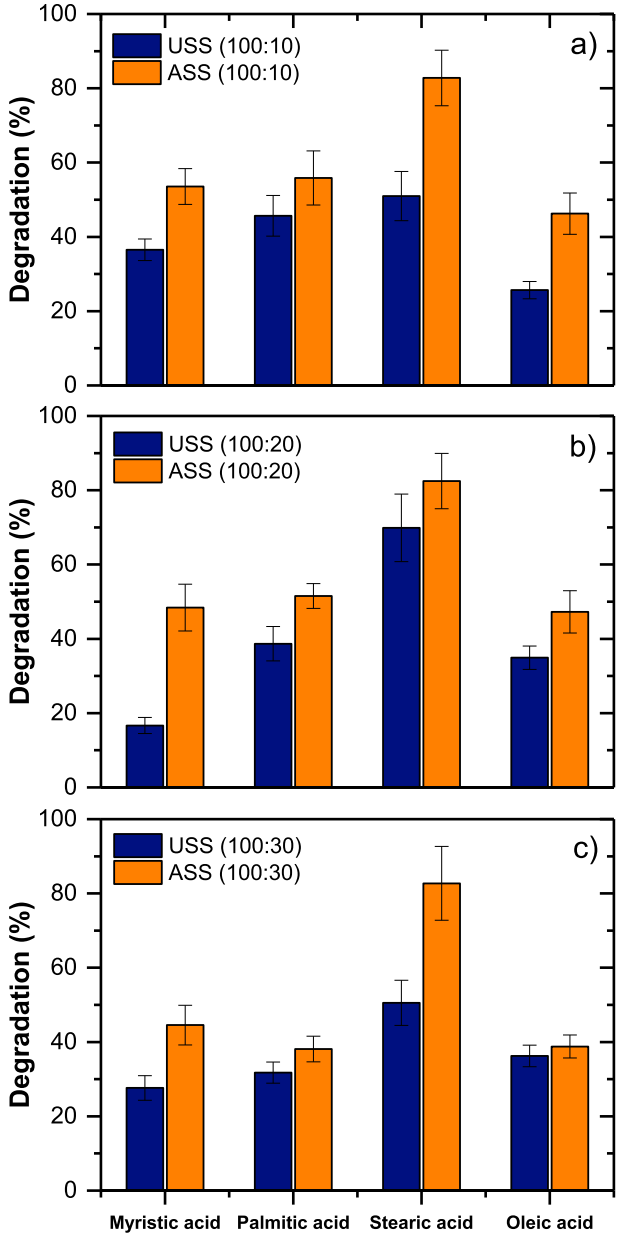

Fig. 3. Profiles of major LCFAs in unacclimatized seed sludge (USS) and acclimatized seed sludge (ASS) digesters fed with 100:10 (a), 100:20 (b), and 100:30 (c).

that acclimation of sludge to LCFA significantly decreased the lag phase of specific methane production. The changes in microbiota in the ASS with acclimatized LCFA-degrading microorganisms, especially syntrophic fatty acid oxidizers would be the one of the major reasons of rapid degradation of LCFAs in the ASS for all studied substrate concentrations corroborating the importance of microbial acclimation to the desired substrate. The microbial dynamics of USS and ASS has been discussed in detail in the later section.

3.4. Influence on microbial community structure due to microbial acclimation to FOG

High throughput 16S rRNA amplicons sequencing revealed the changes in the microbial dynamics of seed sludge because of fed-batch sequential dosage of FOG into the digester. The high diversity between the seed sludge, which was not pre-exposed to FOG (USS), and the ASS was explained by the operational taxonomic units (OTUs). The USS and ASS shared only 7% of the common OTUs, thereby suggesting substantial discrepancies in the variance (Fig. 4a). Numerous phyla with varying dominance were detected in both USS and ASS. Among them, only top 13 major dominant phyla, which conducted critical roles in different phases of anaerobic co-digestion are shown in Fig. 4b. The USS majorly comprised of Proteobacteria (43%) followed by Firmicutes (21%), Bacteroidetes (12%), and Chloroflexi (8%), aggregating 85% of total population. The population of Euryarchaeota was in the normal range (1.4%) for the USS which is in line with the observations reported earlier (Saha et al., 2019a). The ASS mainly comprised of Firmicutes (40%), Bacteroidetes (32%), Synergistetes (10%), and Euryarchaeota (8%), cumulatively forming 90% of the total microbial population. These phyla play a major role in AD, especially in co-digestion of FOG (Kurade et al., 2019; Saha et al., 2019a,b). It has been observed that, the bacterial population belonging to phyla Firmicutes, Bacteroidetes, Proteobacteria and Thermotogae was increased due to addition of oleate in anaerobic reactor (Baserba et al., 2012). Several syntrophic bacteria belonging to Firmicutes are reported to produce volatile fatty acids (VFAs), such as acetic and butyric acids, through hydrolysis of various substrates. Acetic acid is a well-known primary substrate for methane generation through acetoclastic methanogenesis (Yi et al., 2014), while butyric acid is used by some of the genera from Firmicutes (Yang et al., 2014). Bacteroidetes includes various microbial genera that secrete numerous lytic enzymes, such as lyases, hydrolases, lipases, and ligases, which disintegrate complex organic matter to generate acetic acid (Chen et al., 2007). Some of the families in Synergistetes phylum produce acetate, hydrogen, and carbon dioxide via fermentation of glucose and organic acids; however, this occurs only in the presence of hydrogenotrophic methanogens to prevent product inhibition (Si et al., 2016). Similarly, the phylum Euryarchaeota was significantly higher (8%) in the ASS as compared to that in the USS (1.4%), which ultimately resulted in effective transformation of generated acetic acid into methane without inhibition. The phylum Euryarchaeota comprises all the methanogenic bacteria including acetoclastic methanogens, such as *Methanosaeta* and *Methanosarcina,* and hydrogenotrophic methanogens, such as *Methanobacterium*, *Methanoculleus* and *Methanoregula*. Thus, abundance of important phyla of ACoD in ASS provided effective degradation of substrates and generation of methane. An increase in the bacterial population of Synergistetes and Euryarchaeota due to presence of linoleic acid, oleic acid, and palmitic acid in synthetic lipid rich wastewater has been reported recently (Nakasaki et al., 2019).

A genera level analysis was performed and most dominant genera in USS and ASS with a threshold of 1% are shown in Fig. 5. It showed that *Pseudomonas* (32%), *Tissierella* (7%), *Petrimonas* (4%), and *Levilinea* (4%) belonging to the phylum Proteobacteria, Firmicutus, Bacteroidetes, and Chloroflexi, respectively, were highly dominant in the USS. The most dominant genera in the ASS includes *Sporosarcina* (20%) (phylum - Firmicutes), *Proteiniphilum* (11%) (phylum - Bacteroidetes), *Methanosarcina* (8%) (phylum - Euryarchaeota), *Lutaonella* (7%) (phylum - Bacteroidetes), and *Aminobacterium* (6%) (phylum - Synergistetes). Some of the genera belonging to abovementioned phyla perform acetogenesis and interspecies electron transport in the form of hydrogen or formate in syntrophic interactions with methanogens (Saha et al., 2019b). These bacteria oxidize VFAs and alcohols to acetic acid, hydrogen, and carbon dioxide in acetogenesis, which are simultaneously converted to methane by acetoclastic/hydrogenotrophic methanogens. Bacteria in the *Aminobacterium* genus effectively utilize numerous amino acids, thereby producing a range of VFAs and ammonia, especially in the presence of hydrogenotrophic *Methanobacterium* as a hydrogen scavenger (Hamdi et al., 2015; Ferguson et al., 2018). Similarly, *Aminivibrio* (0.32% abundance in ASS) belonging to phylum Synergistetes can oxidize several amino acids in a syntrophic relationship when co-cultured with the hydrogenotrophic methanogen *Methanobacterium formicicum* (Saha et al., 2019b). The genus *Mariniphaga*, a known interspecies electron transporter in anoxic

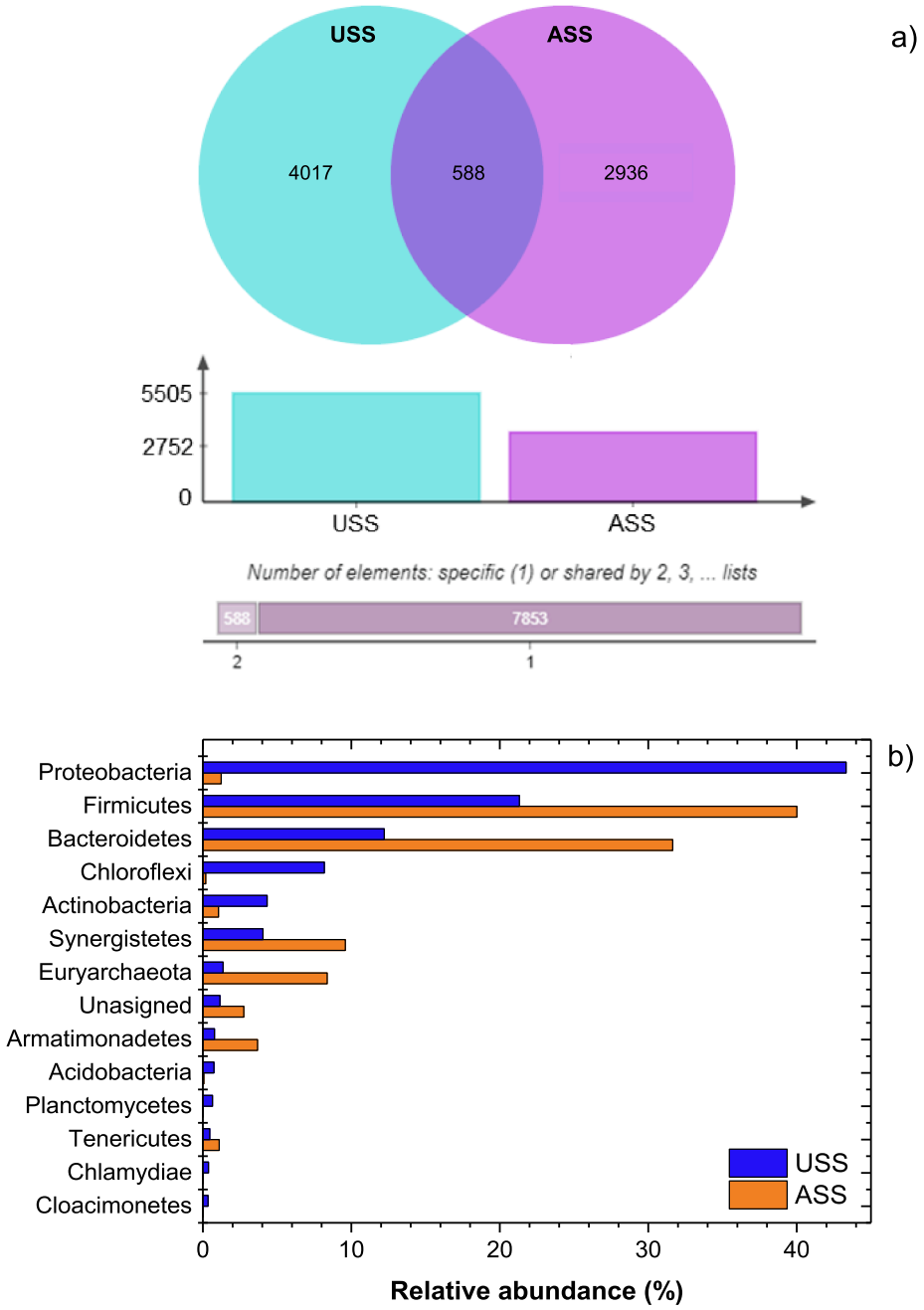

Fig. 4. Venn diagram representing the number of unique and shared operational taxonomic units (OTUs) in unacclimatized seed sludge (USS) and acclimatized seed sludge (ASS) digesters (plotted in MetaCoMET) (a). Microbial community structure of unacclimatized seed sludge (USS) and acclimatized seed sludge (ASS) presented at phyla level (b). High throughput amplicons sequencing of seed sludge was conducted at the beginning of anaerobic co-digestion. For interpretation of the references to high-resolution color in this figure, readers are advised to visit the web version of this article.

environment was 13- fold greater in ASS than USS showing its active role in ACoD. The *Proteiniphilum* genus can ferment organic substrates, such as protein fraction of waste food, to generate numerous VFAs, such as acetate, isobutyrate, propionic acid, isovalerate, and carbon dioxide (Hernandez-Eugenio et al., 2002; Ueki et al., 2014). *Lutaonella* can utilize numerous organic acids and amino acids including pyruvic acid, succinic acid, α-ketobutyric acid, alanine, proline, and leucine (Arun et al., 2009). *Sporosarcina* conducts ureolytic processes (Zhu and Dittrich, 2016); however, its role in AD is not completely investigated. Nevertheless, the fatty acid utilization of *Sporosarcina* is well known. The presence or abundance of these syntrophic bacteria in ACoD suggests their role in direct/indirect electron transfers to methanogens through the oxidation/reduction of conductive materials.

Syntrophomonas was 12- fold higher in case of ASS as compared to that in USS. *Syntrophomonas* is one of the preeminent genera required for the metabolism of LCFAs. Syntrophic fatty acid oxidizers, with association of methanogenic archaea, catalyze the LCFAs into methane through several steps (Sousa et al., 2009). Thus, the dominance of *Syntrophomonas* in the ASS was a major reason for rapid breakdown of LCFAs into acetates, which was further converted to methane through acetoclastic methanogens in a syntrophic relationship. This also avoided the inhibitory accumulation of LCFAs. Ziels et al. (2016) observed that a reactor fed with FOG significantly increased the population of *Syntrophomonas* by 4.7- fold. The FOG-loading capacity of a digester can be predicted by observing the abundance of *Syntrophomonas* population; thus, an improved LCFAs degradation rate can

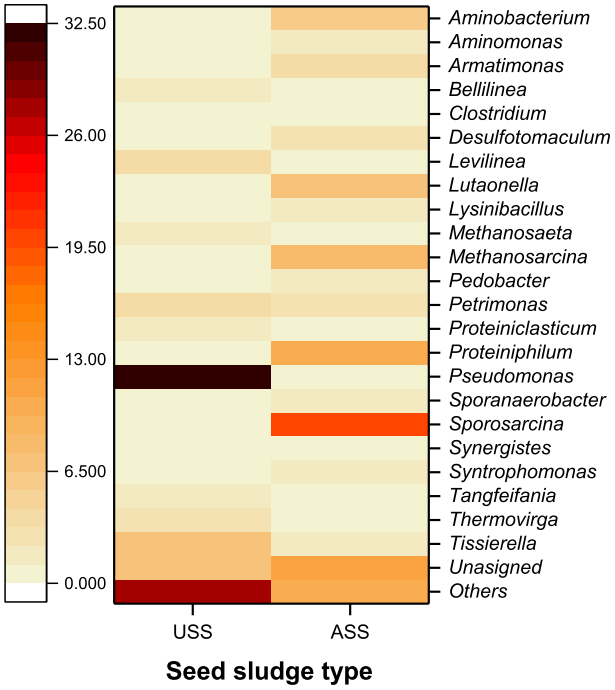

Fig. 5. Heat map of the dominant microbial genera in unacclimatized seed sludge (USS) and acclimatized seed sludge (ASS). Cluster analysis was performed with dominant genera only (abundance threshold was set at 1%). The color intensity of the scale indicates the relative abundance of each genus. For interpretation of the references to high-resolution color in this figure, readers are advised to visit the web version of this article.

be achieved (Ziels et al., 2016; Amha et al., 2017). The syntrophic fatty acid oxidizers and methanogens thrive in a symbiotic relationship, because the former group produce acetate and H_2 and the latter consumes these products to produce methane, which prevents the inhibition of syntrophs by acetate (Smith et al., 2015; Ziels et al., 2016). The methanogenic genera, *Methanosarcina,* was highly abundant (8%) in the ASS along with *Syntrophomonas* as compared to its negligible presence (0.01%) in the USS. *Methanosarcina* usually dominates at higher acetate concentrations (250–500 mg COD L^{-1}) (De Vrieze et al., 2012). The *Methanosarcina* led methanogenesis has been observed for high dosage of acetate (Lins et al., 2014, Nakasaki et al., 2019). These results suggest that microbial acclimatization was crucial for achieving the desired microbial communities for the utilization of a specific substrate and for the impeccable production of methane at a higher rate without causing any system inhibition or failure.

4. Conclusion

The addition of FOG to sewage sludge enhanced methane production in both, USS and ASS digesters. However, gas production in USS showed significantly lower production of methane until the early phase of digestion as compared to the ASS. Higher production of methane in ASS was related to greater degradation of LCFAs, and dominance of phyla Firmicutes, Bacteroidetes, Synergistetes, and Euryarchaeota. The significant abundance of *Syntrophomonas* and *Methanosarcina* genera in ASS supported faster generation rate of methane in an obligatory syntrophic relationship. Therefore, microbial adaptation is advantageous for increasing the efficacy of microbial communities for effective substrate utilization and methane production.

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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