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Composition and Functional Responses of Microbial Community to Temperature and Substrate in Anaerobic Digestion Process

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Abstract

This study investigated the impact of two important key factors including temperature and substrate on microbial community in the anaerobic co-digestion process of septage and longan peel waste by temperature phased anaerobic digesters (TPAD). Denaturing gradient gel electrophoresis (DGGE) and metagenomics sequencing was used to analyzed microbial community structures. The DGGE and cluster analysis results clearly indicated that substrate and temperature strongly influence the structure of bacterial populations. Significant differences of microbial communities were observed from both TPADs digesters. Also, co digestion with longan associated with the changes of bacterial community structure in TPAD system. It was found that *Firmicutes Bacteroidetes Cloacimonetes Tenericutes and Proteobacteria* were most dominant bacterial phyla in TPAD systems. High number of *Firmicutes* and *Tenericutes* were detected from mesophilic tank, while *Bacteroidetes* and *Cloacimonetes* were found from thermophilic reactor. Moreover, each of the digesters harbored distinct yet dynamic microbial populations, and some of the methanogens were significantly correlated with methane productions. *Methanosarcina* and *Methanothermobacter* appeared to be the most dominant methanogenic genera in both digesters operated with different temperatures. The microbiological findings may help understand the metabolism that underpins the anaerobic processes within each of the two digesters of TPAD systems co-digesting septage and agricultural waste.

Keywords : Anaerobic digester; Temperature Phased Anaerobic Digestion (TPAD);
PCR-DGGE technique; Microbial community

Introduction

Anaerobic digestion and its designs including Temperature Phased Anaerobic Digestion (TPAD) have been developed and applied for various types of waste and wastewater including agricultural waste for a source of renewable energy [1-5]. In recent years, the implementation of anaerobic co-digestion has gained a lot of interests due to energy self-efficiency and sustainable waste management [5]. Septage is usually removed from septic tank by vacuum trucks and transport to a distant treatment plant, however, most of the septage is not well treated and mismanaged leading to environmental problems in the country. Consequently, it requires a suitable method for managing and treatment. Considering a large amount of waste and nutrient-rich characteristic, septage has the potential to be used as substrate to produce biogas. Besides, mixing different wastes with septage has been applied for enhancing biogas production. It is due to the supply of missing and imbalance nutrients by the co-substrate and positive synergisms established in the digestion process that support the growth of microorganisms involved hydrolysis and methanogenesis. Previous research have studied using of septage for anaerobic digestion (AD) as treatment and generated renewable energy as co-digestion with landfill leachate [6], food waste [7], and microalgae [8]. In some cases, septage is used as an alternative fertilizer in agriculture and aquaculture without any prior treatment [9].

However, the performance of the anaerobic digestion process (AD) relies on a combination of physical, chemical and biological processes in which microorganisms play an important role. However, the recent system designs based on the information of microbial community composition still have a limited number and remain unclear due to lacking of sufficient detailed knowledge in understanding of the microbial ecology of the system. There are still much left to be known concerning the underlying mechanisms linking operating conditions such as temperature and substrate of AD systems to microbial community structure and function. Therefore, understanding of the complex microbial communities in the AD and their responses to environmental changes might provide the valuable information that can be used to optimize the AD system [10]. Since 99% of bacterial strains cannot be culture-grown on media, assessing the microbial diversity using molecular techniques have more advantages than the conventional method due to it is rapid, less laborious, more sensitive and specific [11-12]. Previously, many studies have been conducted on microbial communities in the temperature phased anaerobic digestion (TPAD) as denaturing gradient gel electrophoresis (DGGE). Yu and co-workers [13] used denaturing gradient gel electrophoresis (DGGE) for the determination of archaeal community and found *Methanobacteria* and *Methanosarcina* from both mesophilic and thermophilic process. Additionally, Hameed and teams [14] determined the microbial communities in TPAD of municipal wastewater sludge and its bacterial community was dominated by *Firmicutes, Bacteriodetes* and *Proteobacteria* while archaeal community was dominated by *Methanimicrobia* and *Methanobacteria*. It is evidenced that core microbial groups have different growth conditions, physiology and stress tolerance which also varies among waste and system condition. Also imbalance among these organisms due to the disturbances which varied from case to case could cause mulfunction of such system. Hence successful TPAD treating

different types of waste requires the study to elucidate the effect of common operating condition on change of microbial community.

This study aimed to investigate the effect of temperature and substrate on the structure of microbial communities involved in anaerobic co-digestion process between septage and longan peel waste using molecular techniques based on DGGE and metagenomic sequencing. This finding could provide more information of the system and could be applied for further enhancement of the AD system treating agricultural waste.

Methodology

Anaerobic co-digestion process and sludge samplings

Laboratory scale of temperature phase anaerobic digestion systems (TPAD) with two reactors operated at 55°C (thermophilic digester) and 35 °C (mesophilic digester) were conducted by Thunyaluk [15] as shown in **Fig. 1**. The TPAD system was consisted of a 30 L-thermophilic (55˚C) and a 10 L-mesophilic (35˚C) reactors. The Organic Loading Rate (OLR) was started from 0.5/1.0 kg VS/m^3-d (OLR of thermophilic/ mesophilic reactor) and sequentially increased to 4.6/5.0 kg VS/m^3-d, respectively. Longan peeling waste was obtained from dried longan production process in Lumphun province and it was used as a co-substrate mixing with septage. Microbial sludge were sampling from untreated septage (ST) and outlet points of both digestors (LP55 and LP35 for thermophilc and mesophilic digester respectively) every week. They were then preserved by mixing with 70% w/w ethanol with the ratio of 1:1. and stored at -20˚C for molecular analysis using PCR-DGGE technique and metagenomic sequencing.

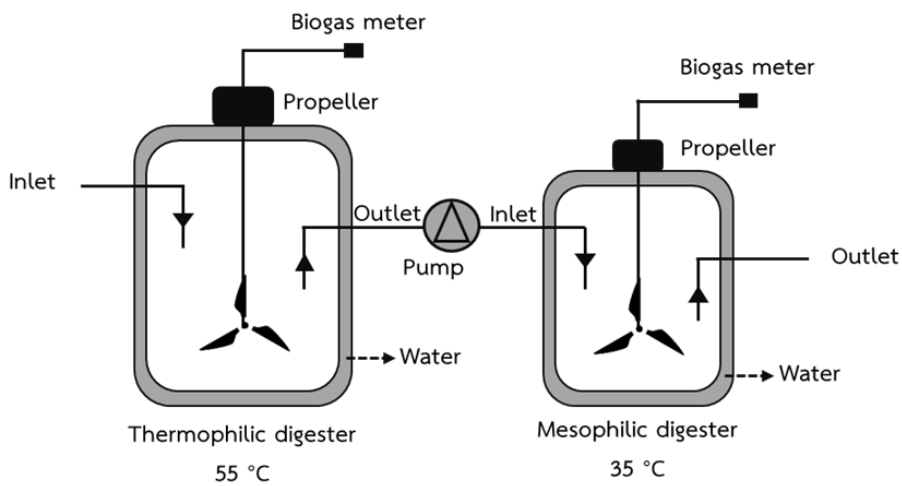

Figure 1 Diagrammatic of temperature phase anaerobic digestion reactor

Microbial community analysis

Total DNA was extracted from the sludge sample using the nucleospin® soil (Bio-rad laboratories, USA) following the manufacturer's instructions. The DNA samples were then sent for metagenomic sequencing analysis which was performed on Illumina HiSeq platform by Novogene Biological Information Technology Co. (Tianjin, China). The relationship of microbial diversity is applied in analyzing the complexity of species diversity for a sample calculated with QIIME (Version 1.7.0), displayed with R software (Version 2.15.3) and generate the Venn and Flower diagram. Unweighted Pair-group Method with Arithmetic Means (UPGMA). Changes of archaea and bacterial communities were analyzed by PCR-DGGE as described by Manatsawat [16] and Pholchan et al [17]. The variable V3 region of 16S rDNA was amplified by using primers targeted to conserved regions of the 16S rDNA gene using specific primers set of 341F/Uni518R [18] and 344F/Arc522R [19]. The amplification conditions used in this study were modified from Hogg and Lehane [20] and conducted using T100 thermal cycler (Bio-rad laboratories, USA). Once the PCR product were stored at -20 °C. DGGE analysis of PCR product was performed on DCode™ system (Bio-Rad laboratories, USA). DGGE images were analyzed via Gene tools analysis software version 3.02.00 of SynGene Genius system (SynGene, UK). Dominant DNA bands representing different bacteria and archaea sequences were excised and purified by RBC TA Cloning Vector Kit (RBC BIOSCIENCE, Taiwan) prior to sequencing (First base, Malaysia) for future study.

Results and Discussions

Performance of TPAD for waste treatment and biogas production

The system performance of anaerobic co-digestion between septage with longan peeling waste are shown in **Table 1**. The results showed that higher biogas production was found from thermophilic digester, while no significant differences ($P \geq 0.05$) of methane contents found from both digesters. Methane production were in the range between 40-56% and 40-58% for thermophilic and mesophilic, respectively. Interestingly, it was found that methane production throughout the operating period from both digesters co-digesting with septage and longan peel waste was more stable than the one feeding with sole substrate (only septage which is explained elsewhere). This possibly could be the result of more complex substances and varieties of substrate composition contained in longan peel waste as co-substrate with septage. Lower COD concentration was found from septage with approximately 11,601.28±9,128 mg/l, while longan peel and septage had higher COD concentration with approximately 38,802±23995 mg/l. Also, high amount of total solid was found from longan peel with the average value of 586,290±24,253 mg/l and C/N of longan peel was about 54%. This is in agreement with some studies suggested that the compositions of the substrates are important for achieving stable processes [21], while low availability of the substrates for microorganisms can be another factor for biomethane production reduction [22].

Table 1 Summary of TPAD reactor performance of anaerobic co-digestion of septage and longan peeling waste

Parameters	Thermophilic digester	Mesophilic digester
pH	7.43 ± 0.28	7.31 ± 0.21
Temperature (°C)	53.62 ± 1.25	35.83 ± 0.87
VFA (mgCH_3COOH/l)	2,020.44 ± 4.88	1,959.10 ± 3.62
Influent COD (mg/l)	38802±23995	46670±8339
Effluent COD (mg/l)	46670±8339	51791±8872
COD removal (% of feed)	27.63±19.02%	18.92±12.92%
Influent TS (mg/l)	85,788±9,637	56,214±10,921
Effluent TS (mg/l)	56,214±10,921	59,273±8,195
TS removal (% of feed)	70.61 ± 1.47	62.86 ± 1.30
Influent VS (mg/l)	76,016±10,477	46,036±8,851
Effluent VS (mg/l)	46,036±8,851	47,791±6,314
VS removal (% of feed)	76.51 ± 1.30	66.35 ± 1.07
Biogas production (ml/day)	10,803.96 ± 41.17	1,730.18 ± 13.99
Total biogas production (ml)	1,761,046.21	282,019.76

Effect of temperature and substrate on relative microbial abundance

The relative abundances of bacterial 16S rRNA transcripts from metagenomic sequencing analysis revealed some differences from the community composition at the DNA level (**Fig. 2**). Firmicutes Bacteroidetes, Cloacimonetes, Tenericutes and Proteobacteria were identified as top dominant phyla within the bacterial community from both digesters and also untreated septage, while their relative abundances varied among samples. This indicated the effect of substrate characteristics and operating condition on microbial community structure. Firmicutes, Bacteroidetes and Proteobacteria were dominated in untreated septage which also appeared in the different abundance ratio in the thermophilic and mesophilic reactors. However, there were some new dominance species harbored in this system. It was found that anaerobic co-digestion decreased the level of Bacteroidetes while increased the level of Firmicutes and relative abundance of Tenericute and Cloacimonetes. This could involve the availability of substances, the metabolic pathway in the biodegradation process and also the operating condition. The results also revealed that high percentage of Firmicutes and Tenericutes were detected from mesophilic reactor, while high ratio of Firmicutes, Bacteroidetes and Cloacimonetes were found from thermophilic reactor. Firmicutes

Bacteroidetes and Proteobacteria have been identified as the main phyla in various AD [23]. Most of members in Firmicutes phylum are syntrophic bacteria that can degrade various VFAs. This coincidence with high percentage of VFA removal in all digesters obtained from this study (50-80%). In addition, Proteobacteria are also important microbes in anaerobic digestion process as they are well-known for glucose, propionate, butyrate, and acetate-utilizing microorganisms [24]. However, all detected bacteria was also able to conduct acidogenesis, which is the second step in the decomposition of organic matter. In addition, Cloacimonetes was decreased only in the mesophilic digester of STLP. However, the relative compositions of these bacteria in digester were variable among different stages of operation which may be associated with the wastewater constituents. According to [25], however, the abundance of phylum Cloacimonetes was linked to lower methane production in the reactors fed with protein-rich substrates. This could explain lower methane production from the digester.

It was found that methanogen communities were unique and diverse between digesters operated with different temperature and substrate variation due to the metabolic pathway occuring in each digesters (**Fig. 3**). The result showed that the euryarchaeota phylum was the most dominant orders. *Methanothermobacter, Methanosarcina, Methanosaeta* and *Methanobacterium* were the top four predominant methanogen genera found from both mesophilic and thermophilic digesters. Some of the methanogens occurring in the system were significantly correlated with methane productions. Some studies suggested that *Methanobacterium* and *Methanothermobacter* was able to grow under high and medium range of temperture, while these species use H_2/CO_2 and formate for methane production [26-27]. Interestingly, *Woesearchaeota* was only found from the thermophilic digester. This species, known as haloarchaea, is distantly related to nitrogen-phosphate remover [28]. This probably linked to large amount of nitrogen-phosphate contained in longan peel in the thermophilic system and may impact on the biological process. In addition, *Methanosaeta* was found to have more population than *Methanosarcina* in septage, whereas *Methanosaeta* was detected less than *Methanosarcina* in TPAD systems co-digested between septage and longan peel. This is because *Methanosarcina* generally have higher growth rates and tolerances against high concentrations of VFA than *Methanosaeta* [29] . From the result, it is likely that some organisms might have only one specific function while some can perform multiple functions such as both hydrolysis and fermentation.

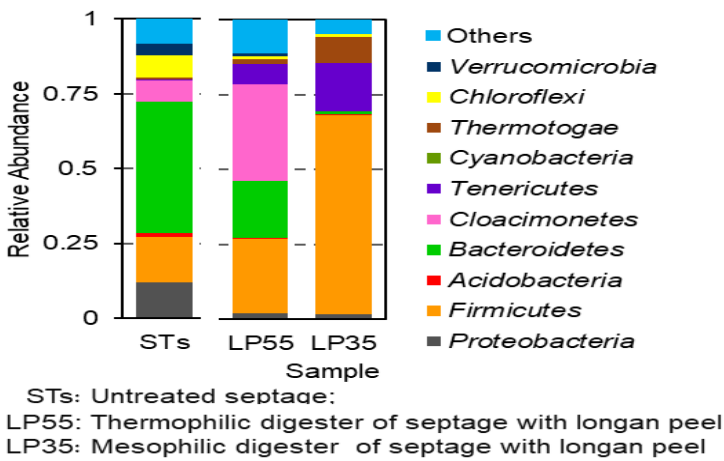

Figure 2 Relative abundance of difference bacteria phylum from TPAD systems at steady state

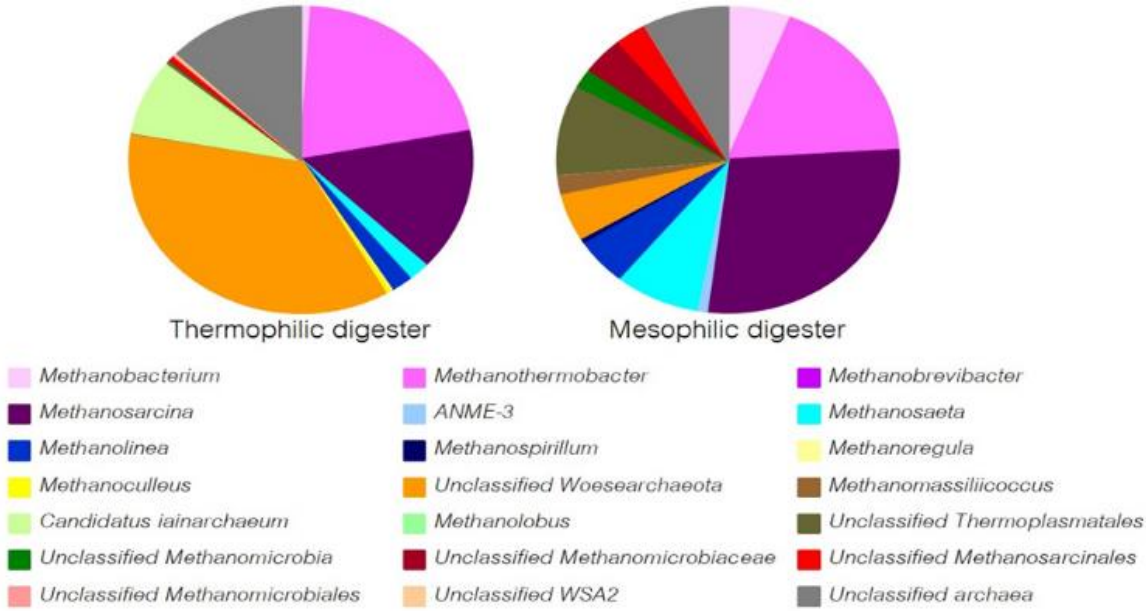

Figure 3 Krona diagram of archaea genera in TPAD system

Effect of temperture and substrate on microbial communities structure

Clustering analysis of bacterial communities from DGGE profile and % relative abundance of dominance species (**Fig. 4 and 5**) showed that thermophilic and mesophilic digester harbored different bacterial communities. There appeared the shifts in the microbial community structure during the operational period. The whole bacterial community and methanogenic community in both digesters formed different groups corresponding to different phase of operation. It was also found that bacterial and archaea community structures were diverse and distinct between digesters due to the variation of substrate and its intermediates and temperature. This coincides with some works reported that substrate variation and temperature had the effect on microbial community structures [30]. Archaea community seemed to be less diverse than bacterial community and the community structure of microbial community in thermophilic reactor showed more stable (**Fig. 5**). This was supported by the Shannon index (*H'*) values which showed that bacterial diversity was higher and have higher similarity than archaea community for both digesters (2.278±0.0605 and 2.268±0.1775 for thermophilic and mesophilic of bacterial community and 1.402±0.092 and 1.589±0.1415 for thermophilic and mesophilic of archaea community). This can be the result of high variety of substrates and intermediate products in the mesophilic phase and there are many different species have the growth condition under this temperature.

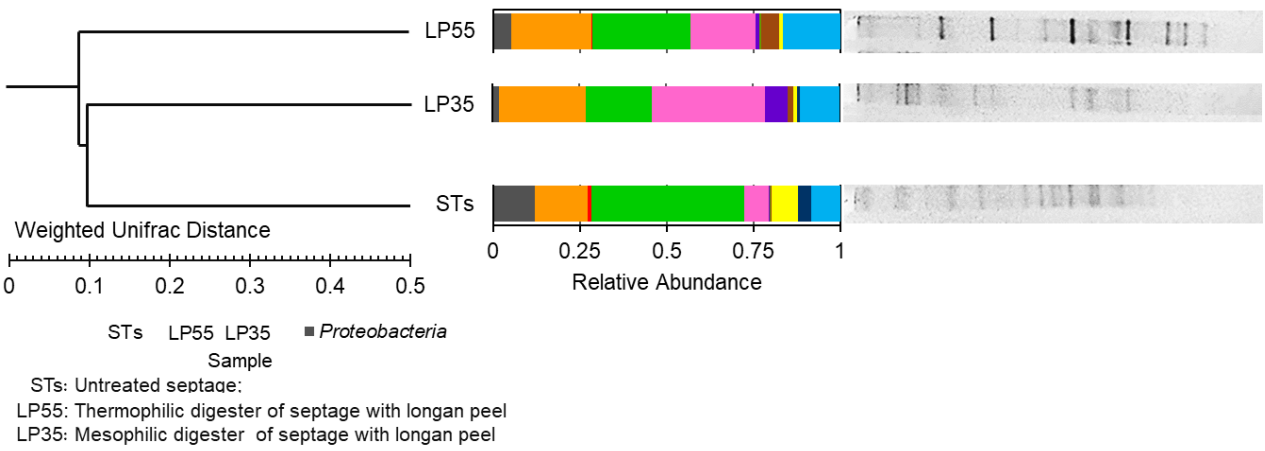

Figure 4 Bacterial DGGE banding pattens from TPAD systems

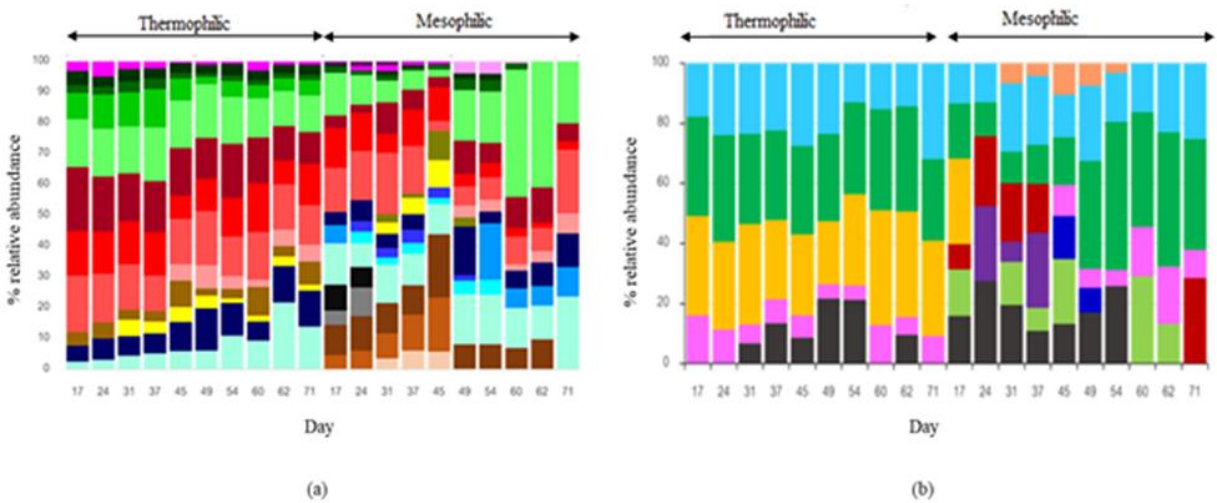

Figure 5 The relative abundance of microbial communities from TPAD system obtained from DGGE profiles (a) bacterial communities (b) Archaea communities

Conclusion

Longan peeling waste and septage were used as the substrate for biogas production in temperature phase anaerobic digestion systems (TPAD) operated under 55°C (thermophilic digester) and 35°C (mesophilic digester). Higher biogas production was achieved from the Thermophilic tank. Each digester harbored distinctive microbial populations, some of which were significantly correlated with the TPAD system performance. The results indicated that *Methanothermobacter* and *Methanosarcina* were the most important methanogenic bacteria, while *Firmicutes Bacteroidetes, Cloacimonetes, Tenericutes and Proteobacteria* were identified as top dominant bacterial phyla from both digesters and also untreated septage. High number of *Firmicutes* and *Tenericutes* were detected from mesophilic tank, while

Bacteroidetes and *Cloacimonetes* were found from thermophilic reactor. Moreover, high ratio of Methanosaeta was found from the system feeding with only septage, whereas it was less detected in TPAD systems co-digested between septage and longan peel. This study proved that substrate and temperture drive the dynamics of key microbial population and its correlation with hydrolytic and metanogenic functionality in the systems.

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References

[1] Adekunle, K. F. and Okolie, J. A. 2015. A Review of Biochemical Process of Anaerobic Digestion. Advances in Bioscience and Biotechnology, 6(3): 205-212.

[2] Han, Y., Sung, S. and Dague, R. R. 1997. Temperature-phased anaerobic digestion of wastewater sludges. Water Science and Technology, 36(6-7): 367-374.

[3] Wang, X., Lu, X., Li, F. and Yang, G. 2014. Effects of Temperature and Carbon-Nitrogen (C/N) Ratio on the Performance of Anaerobic Co-Digestion of Dairy Manure, Chicken Manure and Rice Straw: Focusing on Ammonia Inhibition. PLOS ONE, 9(5): 1-7.

[4] Sung, S. and Santha, H. 2003. Performance of temperature-phased anaerobic digestion (TPAD) system treating dairy cattle wastes. Water Research, 37(7): 1628-1636.

[5] Guo, X., Wang, C., Sun, F., Zhu, W. and Wu, W. 2014. A comparison of microbial characteristics between the thermophilic and mesophilic anaerobic digesters exposed to elevated food waste loadings. Bioresource Technology, 152: 420-428.

[6] Xie, S., Higgins, Bustamante, M.J., Galway, H.and Nghiem, B.L.D. 2018. Current status and perspectives on anaerobic co-digestion and associated downstream processes. Environmental Science: Water Research & Technology, 4: 1759-1770.

[7] Lin, C.-Y., F.-Y. Bian, and Chou, J. 1999. Anaerobic co-digestion of septage and landfill leachate. Bioresource Technology, 68(3): 275-282.

[8] Meghanath, P., Sachin, W., Regina D., Walther, D. and Mutnuri, S. 2015. Anaerobic co-digestion of food waste and septage – A waste to energy project in Nashik city. Carbon: Science and Technology, 7(2): 87-98.

[9] Dingnan, L. and Xiaoqi, Z.J. 2016. Biogas production from anaerobic codigestion of microalgae and septic sludge. Journal of Environmental Engineering, 142(10): 1-10.

[10] Yen-Phi V.T., Rechenburg, A., Vinneras, B., Clemens, J. and Kistemann T. 2010. Pathogens in septage in Vietnam. Science of The Total Environment, 408(9): 2050-2053.

[11] Mata-Alvarez, J., Mace, S. and Llabres P. 2000. Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. Bioresource technology, 74(1): 3-16.

[12] Adzitey, F., Huda, N. and Ali, G.R. 2013. Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. Biotechnology, 3(2): 97-107.

[13] Yu, D., Kurola, J.M., Lähde, K., Kymäläinen, M., Sinkkonen A. and Romantschuk, M. 2014. Biogas production and methanogenic archaeal community in mesophilic and

thermophilic anaerobic co-digestion processes. Journal of Environmental Management, 143: 54-60.

[14] Hameed S.A., Riffat , R., Li, B., Naz, I., Badshah, M., Ahmed, S. and Ali, N. 2019. Microbial population dynamics in temperature phased anaerobic digestion of municipal wastewater sludge. Journal of Chemical Technology & Biotechnology, 94(6): 1816-1831.

[15] Choomjai, T. 2017. Effects of Mixing Ratios and Organic Loading Rates on Biogas Production from Co-digestion of Septage and Longan Peelings by TPAD (master's thesis, Chiang Mai University)

[16] Manatsawa, S. Malem, F., Pholchan, M.K. and Bovonsombut, S. 2017. Detection of bacterial communities in volatile-organic-compound (VOC)-contaminated soil in an industrial estate in eastern Thailand by PCR-DGGE analysis. Chiangmai journal of science, 44: 742-750.

[17] Pholchan, M.K., Baptista, J.D.C, Davenport, R.J. and Curtis, T.P. 2010. Systematic study of the effect of operating variables on reactor performance and microbial diversity in laboratory-scale activated sludge reactors. Water Research, 44: 1341-1352.

[18] Liu H, Tan S., Sheng Z., Yu T. And Liu, Y. 2015. Impact of oxygen on the co-existence of nitrification, denitrification, and sulfate reduction in oxygen based membrane aerated biofilm. Cannadian Journal of Microbiology, 61: 1-6.

[19] Muyzer, G., E.C. de Waal, and Uitterlinden, A.G.1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology, 59(3): 695-700.

[20] Hogg, J.C. and Lehane, M.J. 1999. Identification of bacterial species associated with the sheep scab mite (*Psoroptes ovis*) by using amplified genes coding for 16S rRNA. Applied Environmental Microbiology, 65(9): 4227-4229.

[21] Caporgno M.P., Trobajo R., Caiola N., Ibanez C., Fabregat A. and Bengoa C. 2015. Biogas production from sewage sludge and microalgae co-digestion under mesophilic and thermophilic conditions, Renewable Energy, 75: 374-380.

[22] Wang M., Sahu K.A., Björn R. and Park C. 2013. Anaerobic co-digestion of microalgae Chlorella sp. and waste activated sludge. Bioresource Technology, 152: 585-590.

[23] Zhang, C., Yuan, Q. and Lu, Y. 2018. Inhibitory effects of ammonia on syntrophic propionate oxidation in anaerobic digester sludge. Water Research, 146: 275-287.

[24] Ariesyady, H.D., Ito, T. and Okabe, S. 2007. Functional bacterial and archaeal community structures of major trophic groups in a full-scale anaerobic sludge digester. Water Research, 41(7): 1554-68.

[25] Lebiocka, M., Montusiewicz A. and Cydzik-Kwiatkowska A. 2018. Effect of Bioaugmentation on Biogas Yields and Kinetics in Anaerobic Digestion of Sewage Sludge.International Journal of Environmental Research and Public Health, 15(8): 1-16.

[26] Bryant, M. P. and Boone, D. R. 1987. Isolation and characterization of Methanobacterium formicicum MF. International Journal of Systematic and Evolutionary Microbiology, 37(2): 171-181.

[27] Kotelnikova, S., Macario, A. J. and Pedersen, K. 1998. Methanobacterium subterraneum sp. nov., a new alkaliphilic, eurythermic and halotolerant methanogen isolated

from deep granitic groundwater. International journal of systematic and evolutionary microbiology, 48(2): 357-367.

[28] Kuroda, K, Hatamoto M., Nakahara, N., Abe, K., Takahashi, M., Araki, N. and Yamaguchi, T. 2015. Community composition of known and uncultured archaeal lineages in anaerobic or anoxic wastewater treatment sludge. Microbial Ecology, 69(3): 586-96.

[29] Lv, W., Zhang, W. and Yu, Z. 2013. Evaluation of system performances and microbial communities of two temperature-phased anaerobic digestion systems treating dairy manure. Bioresource Technology, 143: 431-438.

[30] Lv, W., Zhang, W. and Yu, Z. 2016. Volume ratios between the thermophilic and the mesophilic digesters of a temperature-phased anaerobic digestion system affect their performance and microbial communities. New Biotechnology, 33(1): 245-254.