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Feeding frequency efficacy on biogas yield of oily substrate anaerobic digestion in continuous stir tank reactor

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ABSTRACT

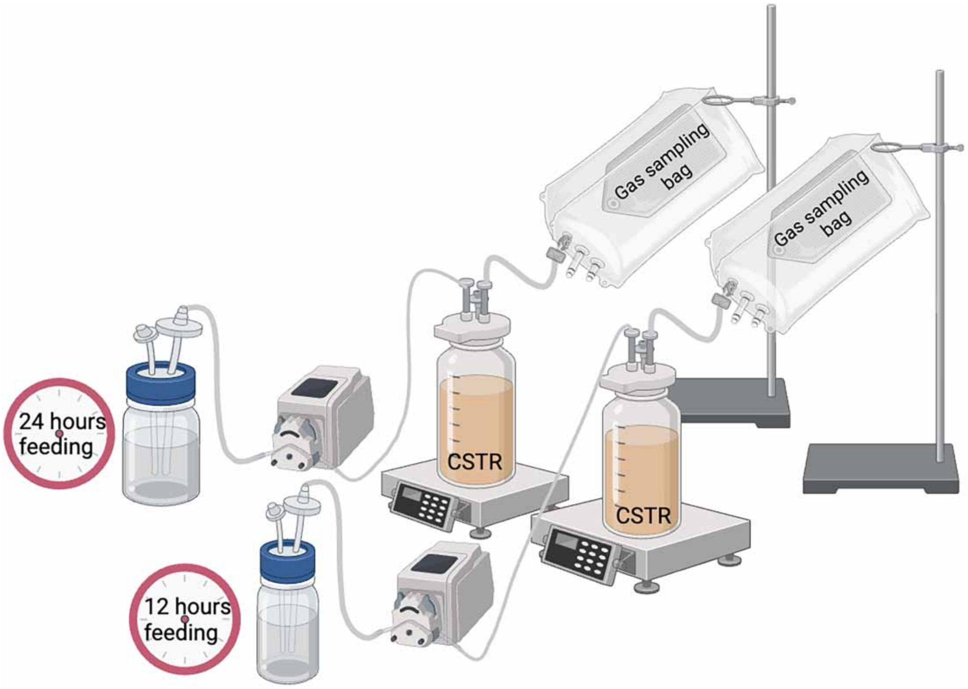
Anaerobic treatment of oily substrate, known as grease trap waste (GTW), was investigated for its practicability via continuous stirred tank reactor (CSTR) at different operating conditions and selected recovery strategies of feeding frequency efficacy. This study determine the performance of feeding frequency efficacy, namely feeding every 24 hours (R_{24H}) and feeding every 12 hours (R_{12H}). Under organic loading rate (OLR) of 2.2 gCOD/L.day, R_{12H} exhibited methane composition of 57%, methane production rate of 0.27 LCH_4/L.day, and methane yield of 0.14 $LCH_4/gCOD_{removed}$. At the same OLR, R_{24H} recorded methane composition of 60%, methane production rate of 0.29 LCH_4/L.day and similar methane yield as R_{12H}. Findings indicated that R_{24H} showed performance comparable to that of R_{12H}. Given minor variation observed in performance, it is recommended that plant operators may consider scheduling two feedings per day for low loading conditions and switch to one feeding per day for higher loading conditions. This strategy is designed to balance the system and prevent shock loads, which could lead to plant shutdowns. This mechanism will induce their conversion to volatile fatty acids (VFAs); thus, reducing the risk of acid accumulation and pH drops, which could inhibit methanogens to produce methane, especially for oily substrate.

Key words: anaerobic digestion, continuous stirred-tank reactor, feeding frequency, long-chain fatty acids, methane, oily substrate

HIGHLIGHTS

- Strategy for lipid inhibition recovery.
- Enhanced methane yield.
- Mono-digestion efficiency strategy.
- Long-chain fatty acids individual profile inhibition.
- Start-up strategy to enhance lipid degradation.

GRAPHICAL ABSTRACT



INTRODUCTION

In Malaysia, there is a strong emphasis on the advancement of anaerobic treatment, specifically within the palm oil industry. Extensive research has been conducted on the anaerobic treatment of palm oil mill effluent as evidenced in studies conducted by Chin *et al.* (2013) and Umar *et al.* (2021). Research on the utilisation of other substrates in Malaysia, apart from palm oil mill effluent, is also growing. For example, the anaerobic digestion process has been studied for its ability to produce biogas from organic fraction of municipal solid waste (Yong *et al.* 2021), landfill leachate (Yusof *et al.* 2022) and food waste (Tanimu *et al.* 2014). Nevertheless, research on the digestion of GTW remains very scarce. In Malaysia, the management of GTW in commercial areas falls under the jurisdiction of municipal council, whereas in domestic areas, it is the responsibility of the national sewerage company. Currently, the treatment of complete GTW material is insufficient. Typically, the oil and grease components are segregated and disposed of as solid waste, while the remaining content is eliminated via drainage. Inadequate maintenance of grease trap has resulted in the accumulation of large amounts of greasy wastewater. If this wastewater enters watercourses, it will have detrimental impacts on human health, watercourses, and environment.

Anaerobic digestion is a promising method for recycling valuable lipids from waste to produce biogas for beneficial purposes. When complex organic substrates are broken down in the absence of oxygen, lipid is a substance that can produce greater amount of biogas with higher concentration of methane compared to protein and glucose (Holohan *et al.* 2022). A comparison was made by Cavaleiro *et al.* (2008) between 1 gm of oleate (lipid) and 1 gm of glucose using standard temperature and pressure (STP) as reference conditions. Methane production was measured to be 1.01 and 0.37 L, respectively. Although lipid hydrolysis shows potential in enhancing biogas production, it can be hindered by the generation of long chain fatty acids (LCFAs) and their destabilisation during the methanogenesis phase, thereby decreasing its biodegradability (Angelidaki & Ahring 1992; Ning *et al.* 2018). Accumulation of oleate and palmitate as LCFA components has been identified as the primary cause of inhibition. It was reported that the anaerobic digestion of food waste (Ren *et al.* 2022), sewage sludge (Martínez *et al.* 2012), and cattle manure (Angelidaki & Ahring 1992; Palatsi *et al.* 2009) was inhibited when their accumulated concentration exceeded 100 mg/L.

However, the utilisation of waste containing a significant amount of lipids is not commonly employed as the primary source due to many inhibitory constraints. For example, waste lipids are excellent co-substrates to improve biogas generation of low-biodegradability substrate. Numerous researchers have demonstrated that co-digestion of waste with high lipid content, such as GTW, with poor biodegradability substrate results in higher biogas generation compared to single substrate. In addition, co-digestion of GTW with sewage sludge resulted in biogas output fluctuating to 68% (Grosser *et al.* 2020), but co-digestion with municipal waste sludge led to 200% increment in daily biogas yield (Shakourifar *et al.* 2020). Although the potential of waste lipid to improve anaerobic co-digestion for biogas production has been known for a while, recent studies aim to further boost lipid degradation, mitigate inhibitory effects, and increase methane production in the mono digestion of waste lipid, such as GTW.

Various techniques can be employed to overcome lipid limitations, including inoculum acclimatisation (Pereira *et al.* 2005), feeding sequence manipulation (Ziels *et al.* 2017), supplements addition (Rasit *et al.* 2018), enzymatic hydrolysis (Mendes *et al.* 2006) and saponification (Battimelli *et al.* 2010). In a previous study, Angelidaki & Ahring (1992) found that shock loads, even at non-inhibitory concentrations, impede reactor activation. To prevent sudden overstressing of the system, the feeding sequence should be thoroughly examined, which allows intermittent feeding into the system. In this situation, LCFAs are consistently provided as frequent feeding to provide the microorganisms sufficient time to adapt and absorb the LCFAs before they are inhibited in the subsequent feeding. Therefore, the impact and excessive loads caused by continual feeding on the cell walls can be minimised (Cavaleiro *et al.* 2020). Previous studies have shown the effects of feeding sequence on the process stabilisation and performance of formulated or synthetic oily waste. Meanwhile, Kang *et al.* (2021) studied constructive findings on methanation and methanogen community structure when utilising dairy cattle manure and lipid-rich food waste leachate, respectively. However, studies on the efficacy of feeding patterns on actual GTW samples in anaerobic treatment are scarcely reported. This study aimed to offer supplementary perspectives on anaerobic waste processing plant which receives various types of waste with occasionally high lipid content. It is crucial to develop an approach to address lipid inhibition and enhance its degradability. This is necessary for process improvement and to mitigate the aforementioned risk.

In sustainability report for the year 2012–2013, Indah Water Konsortium (IWK), a national sewage corporation, reported a total of 20,499 blockages were caused by the deposition of oil and grease in 2013 (IWK 2013). This was the final blockage reported by IWK as the subsequent year, IWK implemented desludging of oil and grease deposits to be used in venturing anaerobic treatment as substrate (IWK 2018). Thus, this study holds great importance in examining the potential treatment strategy of GTW to minimise the amount of waste and transform it into valuable resources for beneficial purposes with limited inhibition. Furthermore, this study also provides additional insights to anaerobic plant operators regarding the management of lipid substrates. The objective of this study was to evaluate the efficacy of anaerobic digestion for GTW treatment by considering the impact of different feeding patterns in continuous stirred tank reactor (CSTR) system.

METHODS

Completely mixed CSTR configuration

Two CSTRs with a working volume of 1 L were utilised, and both reactors were supplied with water jackets to maintain the requisite temperature (35 °C) throughout the entire experiment. A control device was employed to monitor and regulate pH and temperature. The generated biogas was accumulated in 5 L Tedlar gas sample bag. For the feeding frequency strategy, a comparison was made between different semi-continuous feeding modes introduced by Nebot *et al.* (1995), where the total volume designated to be added into the reactors was divided into certain doses, and each dose was added at regular time intervals. Thus, the first reactor, labeled as R_{24H}, was used as control and it was designed for one feeding per day (feeding every 24 h), whereas the second reactor, R_{12H}, was designated as two feedings per day (feeding every 12 h).

Selection of inocula and substrate

Samples of palm oil mill anaerobic sludge (POAS) collected from the settling tank of Felda Serting Hilir Palm Oil Mill was used as inoculum in this study. The samples were stored in tight container at 4 °C before use in the experiments. Table 1 shows the characteristics of the inocula used in both reactors. Based on literature, Khadaroo *et al.* (2020) and Baharuddin *et al.* (2010) also used anaerobic sludge as inoculum for anaerobic digestibility studies. Baharuddin *et al.* (2010) reported that the anaerobic sludge has pH 7.41 and 40.56 g/L COD. In addition, GTW was used as the main substrate in this study. GTW samples were collected from centralised grease trap of food service establishment in Precint 9, Putrajaya, Malaysia. The GTW samples were ground to obtain homogeneous substrate.

Table 1 | Characterization of inocula used in the study

Parameters	Unit	POAS
pH	–	7.35 ± 0.01
TS	g/L	10.45 ± 0.04
VS	g/L	4.86 ± 0.04
COD	g/L	39.53 ± 0.13
Lipid content:		
Myristate C14:0	mg/L	n.d
Palmitate C16:0	mg/L	3.81
Palmitoleate C16:1	mg/L	n.d
Stearate C18:0	mg/L	n.d
Oleate C18:1	mg/L	n.d
Linoleate C18:2	mg/L	n.d

n.d., not detected.

Operating procedure

The operating protocol for both reactors commenced with the start-up procedure and proceeded with the semi-continuous feeding process. At this stage, regular monitoring was performed on pH, alkalinity, chemical oxygen demand (COD), volatile fatty acids (VFA), and LCFAs. This approach has been proven to be effective for initiating anaerobic reactor operation by sustaining active methanogens population engaged in methanogenic activity, particularly when the organic loading is increased during the semi-continuous phase (Griffin *et al.* 1998). For reactor start-up, the biomass was acclimatised with GTW in batch mode before transitioning to semi-continuous feeding.

The first 14 days of reactor operation served as the start-up phase, and the substrate to the inoculum mixture was designed based on volatile solid basis. A ratio of 1 ($VS_{inoculum}$:$VS_{GTW} = 2$ gVS/100 mL) for the mixture were used as the start-up condition as adopted from Griffin *et al.* (1998). At this stage, both reactors were monitored daily for pH, alkalinity, COD, VFA, and LCFAs. The volume of reactants remained constant and was monitored until COD removal was consistent for three consecutive days.

After start-up stage, the reactors were transitioned to semi-continuous feeding mode by gradually increasing the organic loading rate (OLR). Specific operating conditions are presented in Table 2. Reactor performance was assessed based on methane generation, and effluent COD, VFA, LCFA and total solids (TS). The stability of the reactors was evaluated through measurements of pH, total alkalinity, and effluent VFA. The performance and stability of each reactor phase was monitored until equilibrium state was achieved before increasing the OLR. Biogas was collected from connections to both reactors in 5 L Tedlar gas sampling bag.

Table 2 | Operational condition of semi-continuous feeding of R_{R24h} and R_{12H}

Stage	Duration (d)	COD influent (g/L)	Organic loading rate (OLR) (gCOD/L.day)	Hydraulic Retention Time (HRT) (d)
1	27	14.0–14.5	1.3	11
2	19	15.7–16.0	1.6	10
3	16	18.0–18.5	2.1	9
4	15	17.0–17.8	2.2	8
5	14	17.2–17.5	2.5	7
6	14	16.6–16.9	2.8	6
7	13	14.3–14.7	2.9	5
8	13	14.3–14.4	3.6	4

Analysis and calculations

Analytical parameters, including pH, TS, volatile solids (VS), COD, and ammoniacal nitrogen (AN), were assessed for liquid samples according to APHA (1999) guidelines. The biogas samples were analysed using gas chromatography (GC, 6890 N, Agilent Technologies) with HP-Molsive 30.0 m × 530 μm × 50.0 μm nominal column and thermal conductivity detector (TCD) based on the method adopted by Rasit *et al.* (2018). The analysis of VFA was conducted using Thermo Scientific Trace 1300 GC coupled with DB-Wax column and utilised nitrogen as the gas carrier. The samples were prepared for GC injection using the protocol described by Ibrahim *et al.* (2014). Similar to VFA, LCFAs were also examined using GC, utilising HP-Innowax column with dimensions of 30 m × 0.25 mm. A statistical tool, ANOVA (analysis of variance) was used in the study that segments the total variation in a set of data into parts associated with specific sources of variation for testing hypothesis on the parameters of the model used. When there is no significant difference among the selected groups, the null hypothesis is selected, whereas the alternative hypothesis assumes that there is at least one or more than one significant difference among the selected groups.

RESULTS AND DISCUSSION

GTW characterization

Table 3 presents the overall physical and chemical characteristics of GTW, and comparison with the findings of other studies. Essentially, GTW was characterised by its acidity and has a significant proportion of VS/TS. This could be attributed to the elevated lipid percentage, which is associated with high energy content in GTW (Girault *et al.* 2012). Chemical analysis revealed high COD level, indicating significant presence of organic matter. Comparing the COD levels of GTW with those reported in other studies, it was evident that there was significant variability depending on the source of GTW. This range highlighted the diversity of GTW properties and their impact on COD levels. Different industrial processes and food service activities generate GTW with distinct organic loads, leading to varying COD levels. For example, GTW sourced from dairy

Table 3 | Characteristic of GTW used in the study and comparison with other studies

Parameters	**Units**	**GTW from food service area**	**GTW from dairy milk processing plant**	**GTW from wastewater treatment plant**	**GTW from meat processing plant**
Reference	–	This study	Egerland Bueno *et al.* (2021)	Martínez *et al.* (2012)	Neczaj *et al.* (2012)
pH	–	5.84 ± 0.06	n.s	n.s	5.30
TS	g/L	4.23 ± 0.19	0.32 g/g	133.00	n.s
	%	6.41 ± 0.14	n.s	n.s	37.38
VS	g/L	4.10 ± 0.16	0.32 g/g	107.00	n.s
	%	5.91 ± 0.12	n.s	n.s	36.54
VS/TS	%	92	n.s	n.s	98
COD	g/L	64.16 ± 0.09	1.74 g/g	149.00	n.s
O&G	g/L	1.25 ± 0.04	27.6%	n.s	n.s
AN	g/L	0.42 ± 0.01	n.s	n.s	n.s
Elemental analysis	%	$C_{47.46}H_{12.03}O_{39.16}N_{1.17}S_{1.19}$	n.s	n.s	n.s
C/N ratio	–	40.56	n.s	n.s	n.s
C14:0	%	1.95	1.6 g/L	3.7	1.3
C16:0	%	24.98	7.95 g/L	62.60	28.80
C16:1	%	2.91	n.s	n.s	n.s
C18:0	%	15.84	6 g/L	25.0	16.3
C18:1	%	53.66	9.2 g/L	n.s	53.40
C18:2	%	0.66	0.5 g/L	n.s	n.s

C14:0 = Methyl myristate; C16:0 = Methyl palmitate; C16:1 = Methyl palmitoleate; C18:0 = Methyl stearate; C18:1 = Methyl oleate; C18:2 = Methyl linoleate; n.s. = not stated.

milk processing contains different compositions and concentrations of organic materials than those originating from food service areas or wastewater treatment plants. The variability in COD levels among different GTW sources emphasises the importance of source identification and characterisation in waste management and treatment studies. They are reliable predictors of biomass degradation potential in anaerobic digestion. Moreover, high carbon to nitrogen ratio (C/N) of 40.56 was also recorded. According to Gerardi (2003), C/N should be 25:1 for optimum biogas production, and ratio beyond that would cause excessive nitrogen content in the system. However, not all carbon and nitrogen are applicable in the digestion system; thus, the function of C/N ratio could vary considerably. High levels of lipids, COD, and C/N ratio are among the indicators that the breakdown of organic matter is complex and challenging (Gerardi 2003; Long *et al.* 2011; Holohan *et al.* 2022). As a result, LCFAs exert the main inhibitory effect when employing fat-rich waste as the primary material for anaerobic digestion. Thus, the utilisation of GTW is generally avoided due to its negative effect on methane production. Despite the VS/TS value, GTW also has high energy content of 92% (Long *et al.* (2011). This suggests that GTW has the potential to be used as substrate for producing a greater amount of methane, as asserted by Kabouris *et al.* (2009) and Neczaj *et al.* (2012). In addition, anaerobic system is not affected by low ammonia levels as indicated by total ammonia nitrogen (TAN) value, and it is considered a non-contributing element to the inhibitory process.

The chemical formula of GTW determined in this study based on elemental analysis was $C_{1.61}H_{4.87}O$. According to Buswell & Neave (1930), the theoretically expected gas composition of anaerobically digested GTW is 75% methane. At standard temperature and pressure, the theoretical biogas and methane yield obtained in this study were 870 mL biogas/gVS and 650 mL CH_4/ gVS, respectively. The LCFAs based on individual methyl ester showed wide variation in their composition, where the three most prevalent LCFAs identified in the GTW samples were palmitate, stearate, and oleate. This finding is in line with those reported by Egerland Bueno *et al.* (2021) and Neczaj *et al.* (2012). However, individual LCFA showed significant amount of lipids in GTW, as shown in Table 3. Martínez *et al.* (2012) reported that GTW originating from wastewater treatment plant contains high palmitate fraction of lipid. Meanwhile, Egerland Bueno *et al.* (2021) and Neczaj *et al.* (2012) reported that oleate is the most abundant LCFA in GTW originating from dairy processing milk plant and meat processing plant similar to that obtained in this study. In their studies, the potential of GTW to be used as co-substrate to low degradability substrate was investigated. Thus, it can be inferred that the potential of GTW to be used as substrate in anaerobic digestion is significant due to its high organic and energy content. However, the limitation of usage is dependent on the accumulation of LCFAs during the degradation process.

Reactor operation start-up phase

The initial 14 days of reactor operation served as the start-up phase in this study. The duration of the start-up phase was decided based on the observation that the variation in COD removal efficiency should be consistently below 15% for three consecutive days (Griffin *et al.* 1998). This situation can be indicated by the stability of the volatile acid to alkalinity ratio and the increase in methane production prior to introducing fresh OLR into the reactor in semi-continuous feeding mode (Chan *et al.* 2012; Yong *et al.* 2021). Figure 1 depicts the process performance during the start-up process in both reactors. Similar patterns were exhibited by the two reactors in terms of methane generation rate and methane yield, reflecting the trend in methane composition. The R_{12H} and R_{24H} samples exhibited the highest methane production rate of 0.061 LCH_4/L for each. For methane yield, 0.11 and 0.10 $LCH_4/gCOD_{removed}$ were recorded for R_{12H} and R_{24H}, respectively.

Both reactors exhibited lag phase of 9 days, indicating that inhibition occurred due to acclimatisation during the initial stages of the process. During this batch process, the bacteria increasingly acclimate to the new environment, specifically the oily substrate (Lansing *et al.* 2010; Chan *et al.* 2012; Cavaleiro *et al.* 2020). At the beginning of the start-up process, COD removal began slowly until day 9. Rapid COD removal began on day 9–14, ranging from 40 to 60% in both reactors. Low removal efficiency indicated that inhibition occurred due to low toxicity tolerance of the inhibitor. At this stage, the accumulation of inhibitors may reduce the biodegradation rate of organic matter, and thus reduce the conversion to biogas. Thus, the COD removal began slowly and inhibited the process until day 9. This finding is in agreement with that obtained in previous study, where slow COD removal rate was observed in inhibited system with excess oily substrate (dairy milk waste). The slow degradation was recorded for 21 days during the lag phase with low COD removal of less than 50% (Palatsi *et al.* 2011).

The pH stability of the reactors is shown in Figure 2(a), where no significant change in pH was observed in both reactors until day 8. After the adaptation period, substantial pH change was observed on day 9 and became stable until day 14. When the pH value decreased rapidly, it was found that COD removal efficiency, methane production rate and methane yield in

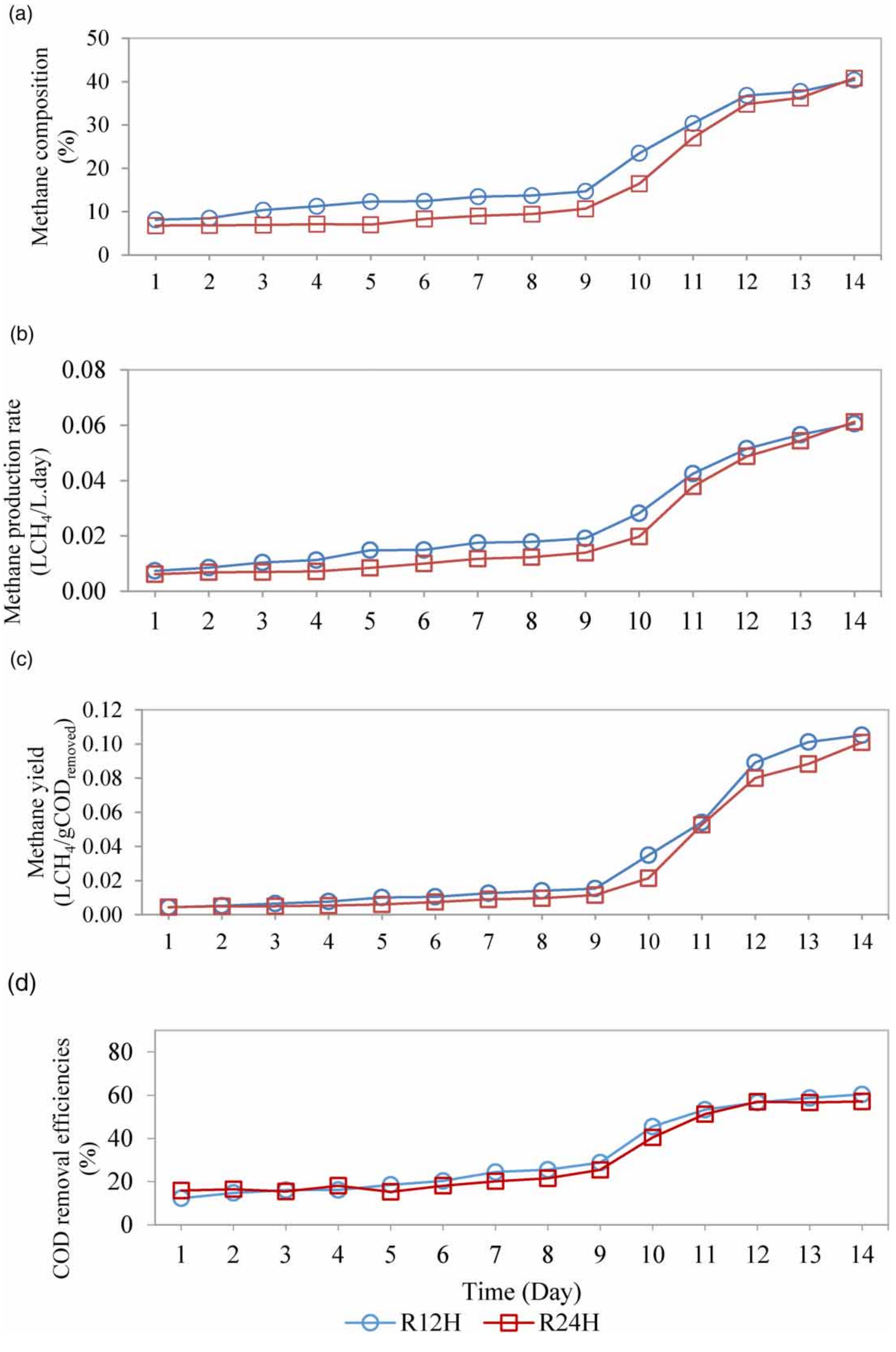


Figure 1 | The profile of (a) methane composition; (b) methane production rate; (c) methane yield and (d) COD removal efficiencies of R_{12H} and R_{24H} during the start-up operation.

both reactors were increased, signifying that active biodegradation rate has occurred in the system (Gonçalves *et al.* 2010; Neves *et al.* 2010; Ren *et al.* 2022).

Figure 2(b) shows the trend of total VFA and alkalinity in both reactors. Basically, alkalinity imparts buffering capability to the reactor to reduce substantial drop in pH (Tabatabaei & Ghanavati 2018). In both reactors, alkalinity values showed an

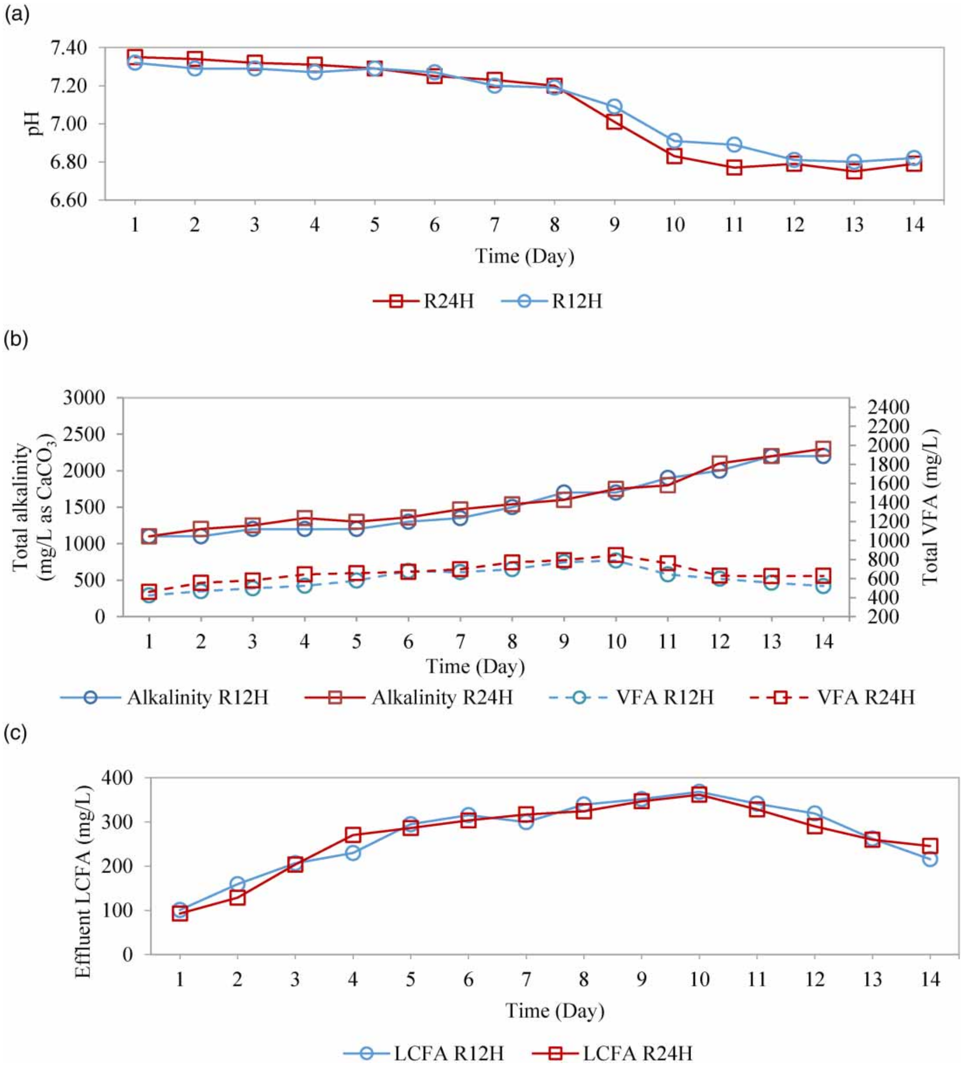


Figure 2 | The profile of effluent (a) pH; (b) Alkalinity and VFA and; (c) LCFA of R_{12H} and R_{24H} during the start-up operation.

increasing trend, reflecting an increase in buffering capacity. The increase in total VFA was accompanied by a decrease in pH in both reactors. After digestion began, maximum VFA accumulation was recorded for the first 10 days. During VFA accumulation, it can be seen that high VFA/TA ratio was recorded by R_{24H} and R_{12H} between 0.42–0.53 and 0.42–0.51, respectively, indicating an imbalance of the anaerobic system. The increase in overall VFA levels and fluctuations in the VFA/TA ratio can be attributed to the prevalence of acid fermentation occurring during the initial phase of reactor start-up (Holohan *et al.* 2022). To maintain a stable digestion system and achieve an equilibrium between the acidogenesis and methanogenesis phases, it is essential to sustain the pH level within the optimal range of 6.8–7.2 to support methanogen activity. Additionally, it is vital to maintain the VFA/TA ratio below 0.3 (Chan *et al.* 2012; Tabatabaei & Ghanavati 2018). Nevertheless, the biomass in both reactors with an extended acid fermentation phase of 10 days could be attributed to poor tolerance level to the inhibitors, resulting in the occurrence of lag phase, as previously observed. Furthermore, the concentration of VFA in both reactors declined after day 10 and remained relatively constant thereafter, indicating that stability was achieved when the alkalinity value increased within the range of 2,000–2,300 mg/L.

LCFA concentration monitoring, as depicted in Figure 2(c), contradicted with that depicted by VFA profile. Variation of LCFA formation in the reactors was observed, with greater LCFA concentrations detected during the start-up phase and increased steadily until day 10, reaching values between 361.54 and 368.11 mg/L in both reactors, respectively. Therefore, the initial period of inactivity observed during the initiation phase may be ascribed to the rapid accumulation of LCFA as suggested by Cavaleiro *et al.* (2020). A delay period of 42 days was reported in batch assay, including treatment of suspended sludge and dairy effluent. During this period, the content of LCFAs reached up to 900 mg/L, and no methane production was seen beyond that value (Cavaleiro *et al.* 2008). The relationship determined between LCFAs and VFA during the start-up phase indicated that while VFA is critical intermediate for methane production, its accumulation, influenced by the presence and breakdown of LCFAs, could lead to system instability. Initially, high LCFA levels contributed to VFA accumulation due to incomplete or inhibited breakdown into VFA. This was evident from the different patterns of LCFA and VFA accumulation in various reactors, with reactors experiencing higher LCFA levels, demonstrating more significant VFA accumulation and subsequent acidification challenges (Cavaleiro *et al.* 2020; Mostafa *et al.* 2020).

Nevertheless, Figure 3 displays the specific characteristics of LCFAs. Oleate accumulation was seen within the initial three days, reaching maximum levels of 148.01 and 133.40 mg/L in R_{24H} and R_{12H}, respectively. Subsequently, the levels of oleate declined in correlation with the elevated concentration of palmitate. The concentration of palmitate began to increase compared to oleate and reached its peak on day 9 in both reactors at 249.78 mg/L (R_{24H}) and 267.89 mg/L (R_{12H}). The main inhibitory substance in the inhibited reactor was palmitate as evidenced by the prolonged accumulation of palmitate and its concentration only beginning to drop after day 10. Nevertheless, no substantial disparity was detected in the myristate and stearate concentrations in both reactors. The concentrations of all acids were initially elevated for several days, and subsequently maintained at a decreasing concentration, remaining relatively constant over the start-up period. Therefore, it can be inferred that the presence and accumulation of stearate and myristate did not inhibit the process.

The breakdown pathway of LCFAs is commonly described as β-oxidation process, where coenzyme A is employed for the oxidation of LCFA (Pereira *et al.* 2004; Holohan *et al.* 2022). According to previous studies, the LCFA toxicity mechanism may be related to the difficulty of the adsorption of active surface onto the biomass cell wall, which affects the overall function of biomass cell transportation and protection (Rinzema *et al.* 1994). This study found that palmitate (69%) and oleate (41%) were the primary LCFAs, which accumulated and remained at higher concentrations. The main product of oleate degradation obtained in this study was palmitate acid, which is consistent with prior research findings. The concentration of palmitate during inhibition was higher than that reported by Martínez *et al.* (2012), ranging from 50 to 100 mg/L when sewage sludge was co-digested with fat, oil and grease. Since the lag phase occurred due to the palmitate accumulation, it can be assumed that the palmitate accumulation rate is greater than the degradation rate, and is the key intermediate in the

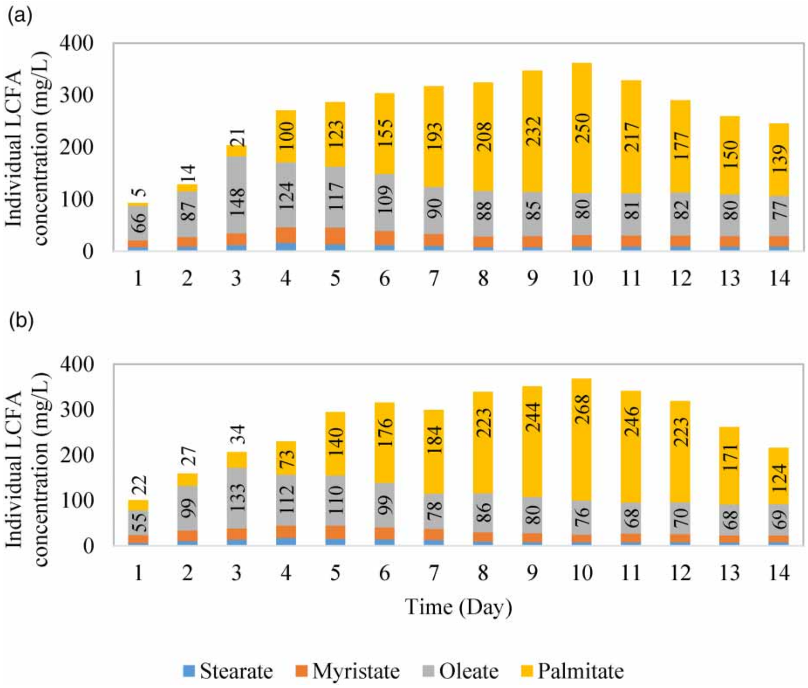


Figure 3 | The individual LCFA profiling of (a) R_{24H} and (b) R_{12H} during the start-up operation.

oleate degradation. After the lag phase, the concentration of palmitate decreased, which in line with the increment of methane production but was consistently in high concentrations. This situation showed that the acclimatisation of biomass in the inhibited reactors was stable, and the biomass developed and acclimatised has better conversion of LCFA into methane, avoiding higher palmitate concentration (Pereira *et al.* 2005; Neves *et al.* 2010).

Semi-continuous feeding operation

Performance of steady-state condition (Figure 4) in both reactors indicated that methanogen activity achieved its maximum efficiency at OLR of 2.2 gCOD/L.day. During this steady-state phase, the highest recorded methane composition was observed in R_{24H} (60%) and R_{12H} (57%). The average maximum methane generation rates measured for R_{24H} and R_{12H} were 0.287 and 0.269 LCH_4/L.day, respectively. In addition, the methane yield of both reactors reached the highest average values at OLR of 2.2 gCOD/L.day, which was consistent with methane composition and production rate performance. The methane production of R_{24H} was 0.14 $LCH_4/gCOD_{removed}$, whereas R_{12H} obtained 0.14 $LCH_4/gCOD_{removed}$. The methane

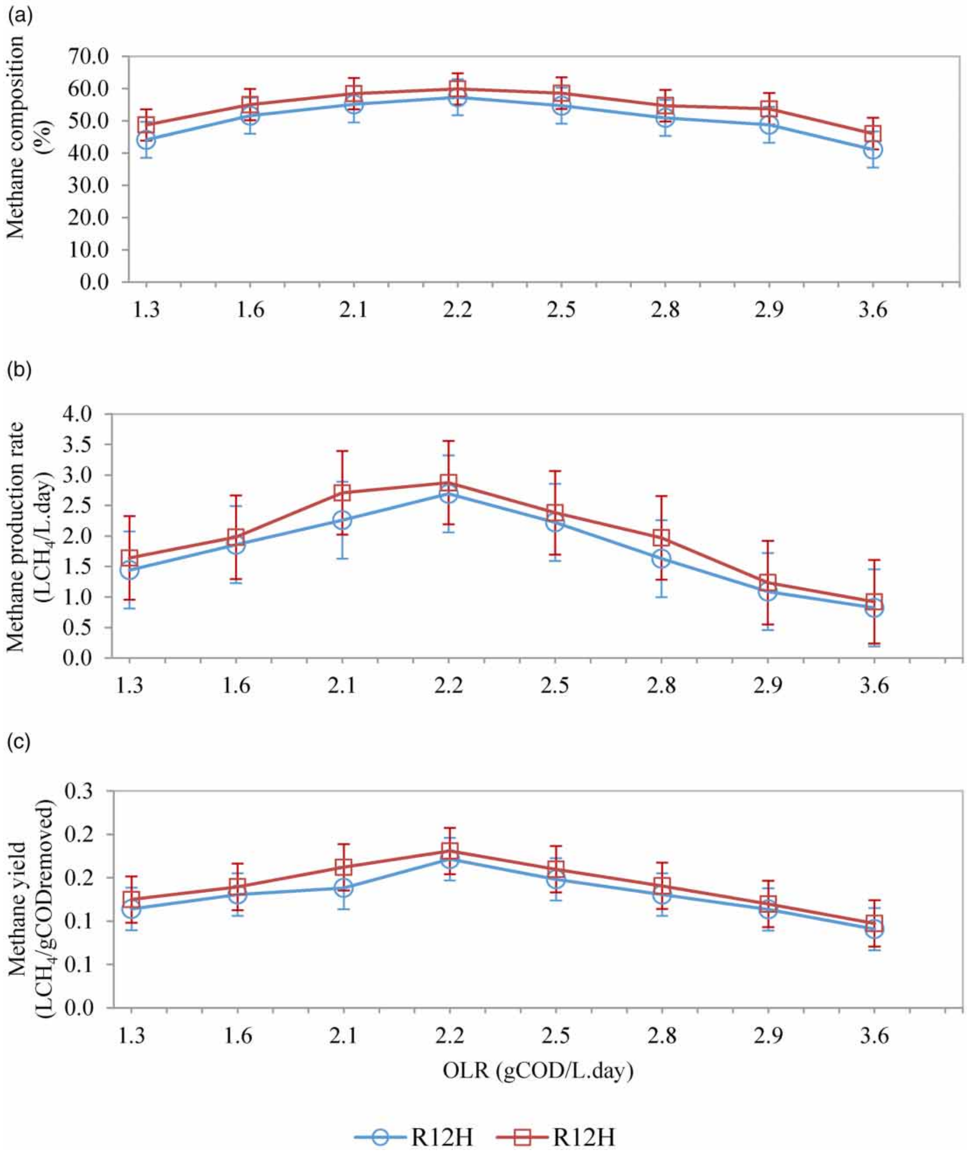


Figure 4 | The profile of (a) methane composition; (b) methane production rate and; (c) methane yield in R_{12H} and R_{24H} during steady state condition.

recovery from COD removal was 88% for R_{24H} and 87% for R_{12H}. The biomethanation process was not completely successful primarily because non-biodegradable substances were collected as sludge flotation, which may prevent the complete breakdown of organic matter (Long *et al.* 2011).

The methane composition, production rate and yield reduced with increasing OLR by 2.5 gCOD/L.day. In both reactors, the methane composition experienced a substantial reduction below 50% until the OLR reached 3.6 gCOD/L.day. In addition to the change in methane composition, the production rate of both reactors showed considerable decline, reaching 0.09 and 0.08 LCH_4/L.day for R_{24H} and R_{12H}, respectively. The methane yield also declined, reaching 0.04 $LCH_4/gCOD_{removed}$ for both reactors. This decrement may be attributed to the removal of organic matter induced by regular feeding without recycling the sludge, which could lower the ability to tolerate the substrate (Lin *et al.* 2011; Long *et al.* 2011).

The VFA values in both reactors began to increase when OLR was 2.5 gCOD/L.day and beyond. The corresponding alkalinity was also reduced to less than 1,000 mg/L in both reactors at the end of the experiments. The VFA/alkalinity ratio also showed unstable condition when it was greater than 0.3, leading to reactor upset, and the final VFA values for both reactors were 592 and 556 mg/L for R_{12H} and R_{24H}, respectively. During the instability period, pH adjustment and alkalinity supplementation did not improve the conditions in both reactors, indicating that acid-forming bacteria hindered methanogenic activity.

Amidst the disruption of both reactors, careful examination revealed the presence of sludge flotation. This situation is in agreement with the findings reported by Jeganathan *et al.* (2006). Their study found that when oily wastewater from the food sector was used at higher OLR of 5.2 gCOD/L.day, up to 75% of the COD was degraded. The methane yield was measured as 0.24 $LCH_4/gCOD_{removed}$. However, there was a notable sludge flotation, which ultimately lowered the overall degradation to 40%.

Figure 5 depicts the reactor stability during steady-state performance. After acclimatisation period during start-up process, the pH values of R_{24H} increased and maintained within 7.03–7.12 throughout the OLR of 1.3–2.2 gCOD/L.day. This indicated stable operating conditions in the reactor. For R_{12H}, the pH values were fluctuated but maintained within favourable conditions for methanogenesis. As a result, a decline in pH was seen as the OLR increased in both reactors.

The instability of the reactor proved to be due to the occurrence of rapid VFA accumulation towards the end of the experiments. The pH levels in both reactors reached a minimum of 6.6 at the end of the experiments, indicating unfavourable conditions for methanogenesis. This was directly related to the disruption of reactor performance. After initial increment during start-up, the VFA concentration decreased, indicating substrate degradation, and then stabilised until OLR of 2.2 gCOD/L.day. Low VFA/alkalinity ratio (<0.3) indicated that the reactors were in stable condition. However, the lowest performance of methane production was likely due to VFA instability for R_{12H}, where the VFA/alkalinity ratio was found to be more than 0.3 for the first OLR. Then, the reactors stabilised towards OLR of 2.2 gCOD/L.day. Throughout the stable period, the alkalinity and VFA values remained within the ideal range for methanogens to function efficiently. The VFA values in both reactors began to increase when OLR reached 2.5 gCOD/L.day, whereas alkalinity decreased to less than 1,000 mg/L at the end of the experiments. Unstable state was observed when the VFA/alkalinity ratio exceeded 0.3, resulting in reactor upset. Overlapping error bars indicated that there was a slight difference between the experimental values of both reactors. Throughout the instability period, attempts to modify the pH and supply alkalinity did not lead to improvement in both reactors. This suggested that the acid-forming bacteria successfully outcompeted the methanogens. The VFA and alkalinity in both reactors demonstrated the same trends during semi-continuous feeding operation except that higher and unstable concentrations were recorded for R_{12H}. Therefore, the overall methane production performance remained slightly lower in R_{12H} when compared to R_{24H}. This could be influenced by the VFA instability, indicating that frequent feeding (two feedings per day) may reduce the oxidation duration and accumulation sustained in higher concentrations throughout the experiments. Thus, reducing the feeding frequency will enable further oxidation of VFA, and thus enhance reactor performance (Gonçalves *et al.* 2012; Ziels *et al.* 2017).

The overall LCFA profile in both reactors showed the same trend as that shown by VFA profile. After acclimatisation period and lag phase during the start-up process due to inhibition by LCFAs, the biomass in both reactors demonstrated the ability to degrade LCFAs. The concentration values remained consistent, without significant difference observed between the two reactors (indicated by overlapping error bars) until reaching OLR of 2.2 gCOD/L.day. At this OLR, values below 170 mg/L were consistently recorded. This suggested that methanogenic activity in the acclimated environment exhibited both an increase and stability in tolerance. Biomass experienced lag phase during start-up but eventually became tolerant and acclimated to LCFAs. The acclimated biomass could improve the conversion and recovery of LCFAs afterwards

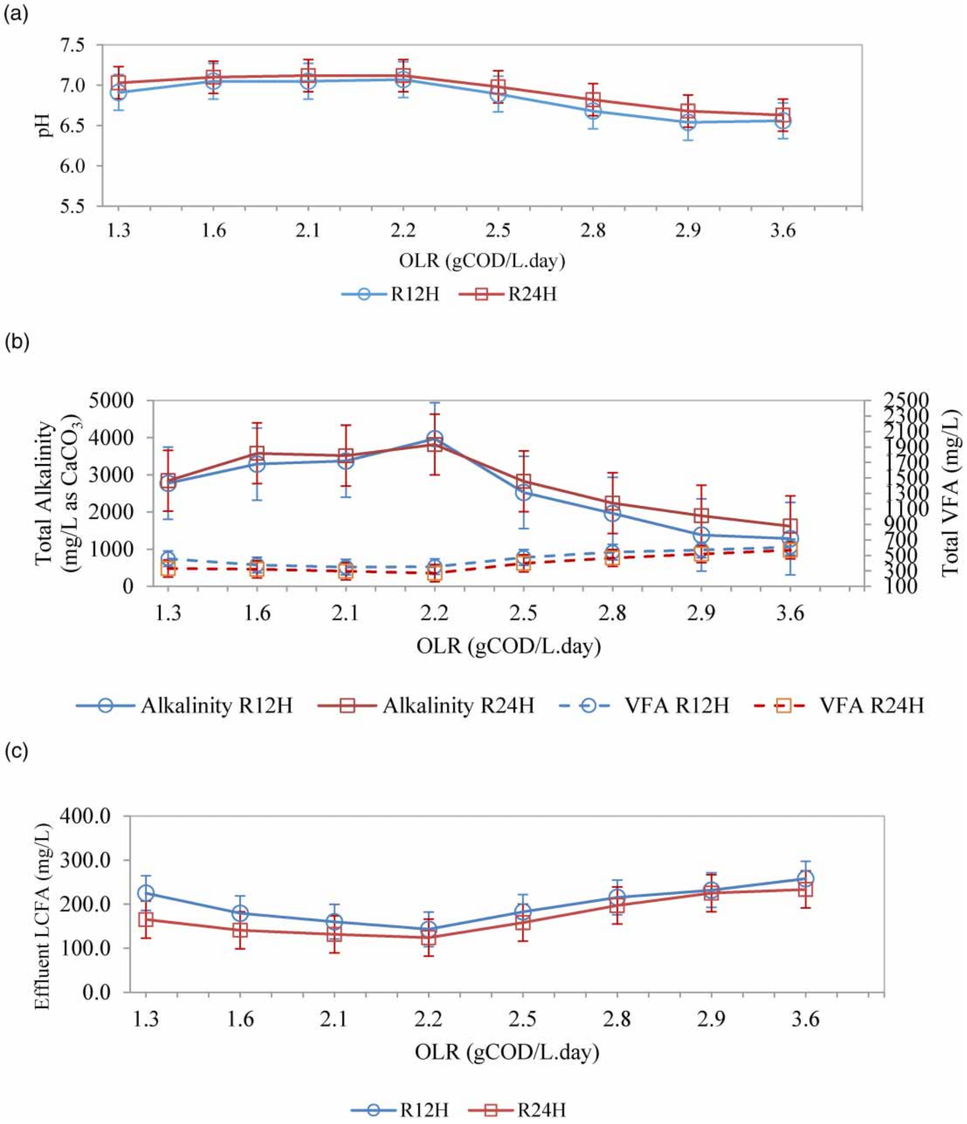


Figure 5 | The profile of (a) pH; (b) total alkalinity and VFA; and (c) LCFA in R_{12H} and R_{24H} during steady state condition.

(Neves *et al.* 2010; Holohan *et al.* 2022). This indicated that the LCFAs were partially degraded before introducing new feeding. The least feeding frequency (one feeding per day) for R_{24H} promoted longer oxidation and mineralisation period of accumulated LCFAs. This hypothesis is contrary to the findings of other researchers. For instance, Gonçalves *et al.* (2012) asserted that reducing the feeding frequency from continuous feeding to 1 time feeding per day is a good strategy for anaerobic digestion of olive mill wastewater. It indicates that there is less tolerance towards the accumulation of LCFAs when less frequent feeding frequency is applied (Gonçalves *et al.* 2012; Ziels *et al.* 2017; Holohan *et al.* 2022). In fact, Gonçalves *et al.* (2012) also concluded that oleate and palmitate are the main LCFA compounds, which accumulated in high concentrations as the feeding frequency increases. The reduction in feeding frequency also reduced LCFA increment and increased the methane production by 70%. Nevertheless, LCFAs began to accumulate again as the OLR increased to 2.5 gCOD/L.day and up to 3.6 gCOD/L.day. This is in agreement with that observed in the VFA concentration, leading to digester upset. In addition, the type of substrate can significantly influence the change in feeding frequency, which also affects anaerobic digestion performance. In particular, different substrates may require specific adaptations in feeding frequency to optimise LCFA degradation and methane production. For instance, substrates that are easily degradable and have low lipid content may perform well under more frequent feeding regimes without risking significant LCFA accumulation. Understanding

Table 4 | Performance comparison for frequent feeding, R_{12H} and R_{24H} used in other study in terms of methane production and lag phase occurrence

System	Substrate	Feeding patterns	Organic loading rate (gCOD/L.day)	Methane yield ($LCH_4/gCOD_{removed}$)	Reference
CSTR	Grease trap waste	1 time feeding/day 2 times feeding/day	2.20	0.14 0.14	This study
CSTR	Cow dairy manure	1 times feeding/ 2 days Continuous feeding	2.20–3.00	290* 200*	Ziels *et al.* (2017)
CSTR	Chicken litter slurry	1 time feeding/day 6 times feeding/day	n.s	17.1** 20.5**	Bombardiere *et al.* (2007)
CSTR	Laminaria Japonica (marine algae)	1 time feeding/day 2 times feeding/day	n.s	0.20** 0.25**	Piao *et al.* (2013)
UAB	Olive mill wastewater	1 time feeding/day Continuous feeding	1.00 1.90	0.75 0.20	Gonçalves *et al.* (2012)
TAF	Food industry wastewater	1 time feeding/day 6 times feeding/day	4.01 3.60	0.27 0.25	Nebot *et al.* (1995)

*, measured as biogas production (mL CH_4/gVSfed); **, measured as biogas production (m3/day); TAF, thermophilic anaerobic filter; CSTR, continuously stir tank reactor; UAB, up-flow anaerobic bioreactor; TAF, thermophilic anaerobic filter; n.s, not stated.

these relationships can help in designing more efficient and stable anaerobic digestion reactors tailored to the specific characteristics of the substrate.

Table 4 summarises the performance of methane production in frequent feeding reactors, namely R_{12H} and R_{24H}. The overall methane production in R_{12H} remained low with the same value as that in R_{24H}. This situation indicated that one feeding per day in R_{24H} has performance comparable to two feedings per day in R_{12H}. This finding is similar to that of Ziels *et al.* (2017) but contradicted other previous study. It has been suggested that increased feeding frequency may increase the tolerance to the microbial community when low loading is fed in frequent feeding capacity (Bombardiere *et al.* 2007; Piao *et al.* 2013). High tolerance to microbial digestion activity will improve methane yield. However, when it comes to the degradation of high lipid content substrate, it is suggested that the tolerance of accumulated LCFAs is more acceptable when less frequent feeding frequency is applied (Pereira *et al.* 2005; Gonçalves *et al.* 2012; Holohan 2020). As supported by Gonçalves *et al.* (2012) and Nebot *et al.* (1995), a longer cycle provided by one feeding per day promotes higher methane production for high lipid waste. These results suggested that reducing the feeding frequency would reduce the instability of the reactor as observed in VFA and LCFA concentrations when accumulated LCFAs experienced longer oxidation and mineralisation; thus, biodegradation and methane yield are enhanced (Nielsen & Ahring 2006; Gonçalves *et al.* 2012). Furthermore, since slight performance difference was observed between R_{12H} and R_{24H}, it can be suggested that whenever plant operators receive excess oily wastewater, they can allow two feedings per day in their daily operation to reduce the wastewater capacity to be treated.

One-way ANOVA was used in this study to validate the experimental data. Analysis was conducted on the biogas production rate and yield for both start-up and steady-state operations between two groups, namely R_{12H} and R_{24H}. The null hypothesis was denoted as no significant difference between the data. If the observed F_{value} is greater than $F_{critical}$ and the probability (P-value) is smaller than 0.05, the null hypothesis is rejected. This means that the data have significant differences between the mean of the groups. In summary, this study obtained F_{value} lower than $F_{critical}$ and P_{value} greater than 0.05, implying that there was no significant difference between the two groups (R_{12H} and R_{24H}) in both operations; thus, the null hypothesis was accepted.

CONCLUSIONS

The strategy of increasing the feeding frequency to two feedings per day (R_{12H}) in anaerobic digestion of GTW did not enhance methane production compared to one feeding per day (R_{24H}) when using OLR of 1.3 and 3.6 gCOD/L/d in CSTR. The findings demonstrated comparable performance for both reactors. During the start-up stage of both reactors, lag phase occurred for 9 days. The overall performance of R_{12H} during this stage resulted in methane composition of 57%, methane production rate of 0.27 LCH_4/L.day, and methane yield of 0.14 $LCH_4/gCOD_{removed}$ under OLR of 2.2 gCOD/L.day. At the same OLR, R_{24H} recorded methane composition of 60%, methane production rate of 0.287 LCH_4/L.day, and methane yield of 0.14 $LCH_4/gCOD_{removed}$. This performance was supported by the VFA and LCFA concentrations observed in the reactors. On the other hand, the strategy of reducing feeding frequency provided sufficient time for LCFA oxidation and mineralisation. Thus, toxicity effects from LCFA accumulation could be reduced. Since slight performance difference was visible between R_{12H} and $R_{24H\ 1}$, it is suggested that whenever plant operators receive excess oily wastewater, they can allow two feedings per day in their daily operation to reduce the wastewater capacity to be treated.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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