Assessment of multiple anaerobic co-digestions and related microbial community of molasses with rice alcohol wastewater

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Research

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Abstract

Background

Molasses is a highly dense and refined byproduct produced in the sugarcane industry, and it contains high amounts of degradable compounds. These compounds can potentially be converted into renewable products biologically. However, the involved biological process is negatively influenced by the high chemical oxygen demand (COD) of molasses and its high ion concentration, although this problem is commonly addressed by dilutions.

Results

The co-digestion of molasses with rice alcohol waste water (RAW) was compared with its mono-digestion at an increasing organic loading rate (OLR). Both processes were assessed by detecting the COD removal rate, the methane contents of biogas, and the structure and composition of microbial communities at different stages. Results showed that the co-digestion is stable up to a maximum OLR of 16 g COD $L^{-1}d^{-1}$. By contrast, after the acclimatization phase, the mono-digestion process was upset twice, which occurred at a maximum OLR of 9 and 10 g COD $L^{-1}d^{-1}$. The co-digestion procgess demonstrated consistency in terms of COD removal rates (86.36% ± 0.99–90.72% ± 0.63%) and methane contents (58.10% ± 1.12–64.47% ± 0.59%) compared with the mono-digestion process. Microbial community analysis showed that the relative abundance of bacterial and archaeal communities differs between the processes at different stages. However, in both processes, *Propionibacteriaceae* was the most abundant family in the bacterial communities, whereas *Methanosaetaceae* was abundant in the archaeal communities.

Conclusion

Rice alcohol wastewater could be a good co-substrate for anaerobic digestion of molasses. Integrate molasses into progressive biogas production at high OLR.

1. Background

Side streams produced in huge quantities in agro-industrial processes contain high organic compounds of low value, and these streams are an attractive feedstock to be used in bio-refineries. The highly biodegradable substances present in these streams can be converted into chemicals of interest [1, 2, 3]. Among the agro-industrial byproducts, sugarcane-derived products are potential sources of high-value compounds, such as biomethane, biohydrogen, and organic acids, which are obtained through fermentation [4]. During anaerobic digestion, a sufficient glucose or sucrose concentration is required for the growth of microbes that facilitate the generation of byproducts, such as methane, hydrogen, and

organic acids [5]. Molasses, which is composed of approximately 45% sugar, is a viable raw material for biofuel production through fermentation [6].

An important organic agro-industrial byproduct, molasses is a highly dense material produced through the crystallization of sugarcane [7]. It is also considered a major raw material for biogas production due to its high organic fraction content [1, 8]. However, despite the increasing interest molasses receives and despite its high organic fraction content, its anaerobic digestion is limited due to its high COD, its high ion concentration, and its melanoidin content; therefore, molasses should be diluted strongly before it can be used in any biological process [9, 10, 11].

The high organic fraction of molasses is attributed to its high sucrose content, and sucrose can be used as a substrate in bio-refineries and is possibly involved in a number of microbial pathways [2, 12]. Biofuels, such as ethanol, hydrogen, and methane, are well known products of molasses digestion [13]. Moreover, products such as alcohol, amino acids, and baker's yeast are produced from molasses [1].

Anaerobic digestion is an effective, widely acceptable, and cost-effective technology to degrade organic matter to produce mainly methane and carbon dioxide. It is a multistep process usually involves four groups of microorganisms that drive hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Methanogenesis, the last step, is considered to be the most critical part of the process with respect process failure due to variations in process conditions (temperature, pH, OLR and alkalinity) [14, 15, 16, 17, 18]. Therefore, depending on these conditions, stable process operation could only be possible when they co-exist in digesters. When the conditions fluctuate, a shift in either of the step could cause process failure [19]. Many industrial wastes have been extensively treated through anaerobic technologies [20, 12]. Anaerobic co-digestion describes the concurrent digestion involve multi-substrates, allows to enhance biogas production. Co-substrates provide additional micronutrients in the system, and these micronutrients are vital in enhancing the fermentation facilitated by a microbial community [21, 22]. Co-digestion, nowadays attains much attention in several countries with respect to the smooth performance of the process.

An imbalance in microorganisms, such as methanogens and other microbes, upsets the fermentation process [23, 24, 25]. Therefore, evenness in the microbial community is the most critical factor in ensuring stable fermentation [26]. The stability and efficiency of fermentation are highly dependent on the mutual and syntrophic relationship of different microbial groups responsible for hydrolysis, acetogenesis, acidogenesis, and methanogenesis [27, 28]. A balanced composition of methanogens and other microbes ensures the stability of the fermentation process. Highly active microbes in the acedogenic and acetogenic phases resulted in high VFA production, which causes the process to fail [29, 14]. Hence, the functional stability of the process is highly associated with the evenness of the microbial community [30]. Most importantly, the microbial balance has been shown to enhance methane production [28].

Keeping in view the afore-mentioned importance the research work was design to evaluate anaerobic co-digestion of molasses with rice alcohol wastewater and to compare with mono-digestion of molasses in addition to analysis of microbial composition. The efficiency of both processes was assessed in terms of

COD removal rate and percent methane contents of biogas produced. Additionally, VFAs were also emphasized periodically since these could negatively affect the digestion process. While an NGS based technique was applied to identify and characterize the Archaeal and bacterial communities in different phases of the process.

2. Results And Discussion

2.1. Impact of operating conditions on COD removal

2.1.1. Mono-digestion and co-digestion process

In the current study it was observed that in the mono-digestion process (Fig. 2b, Phase I) the COD removal efficiency was gradually increased up to 89.31 ± 0.24% with increasing OLR to 7 g COD L^{-1}. d^{-1}. However, in phase II when OLR was increased to 9 g COD L^{-1}. d^{-1}, a dramatic decrease in COD removal rate was noted (82.56 ± 0.85%) and continuously decreased to 74.96 ± 1.28% (Fig. 2b). This inhibition at maximum OLR could be the accumulation of high VFA contents results in dropping the pH values of the system or could be the high loading rate which might be intolerable by the methanogens in the digester [31, 32, 33]. After the digesters sour at 9 g COD L^{-1}. d^{-1}, feeding was stopped for 4 consecutive days to stabilize the process. The process was resumed with feeding low OLR (4 g COD L^{-1}. d^{-1}) along with the addition of co-substrate (RAW with molasses). This feeding of low OLR from 4–7 g COD L^{-1}. d^{-1} and co-substrate stabilized the process and the COD removal rate was then recorded as 83.91 ± 1.25% to 91.12 ± 0.05% (Fig. 2b, Phase III). In phase IV, when only molasses were fed in the digester, the COD removal rate was ranged from 87.56 ± 0.69% to 89.29 ± 0.094% during a stepwise increase in OLR up to 10 g COD L^{-1}. d^{-1}. However, the efficiency of the digester decreased to 70.09 ± 1.53% by continuous addition of OLR up to 10 g COD L^{-1}. d^{-1} (Fig. 2b).

In the present study, mono-digestion of molasses up to OLR (8 g COD L^{-1}. d^{-1} and 10 g COD L^{-1}. d^{-1}) could be attributed to the stable microbial communities in the sludge after a co-digestion phase I & III (Fig. 2b). Our results are consistent with De Vrieze et al. [14] and Detman et al. [8] regarding the COD removal rate. However, De Vrieze et al. [14] reported a rapid decrease in COD removal rate at OLR of 4.1 g COD L^{-1}. d^{-1}. High strength molasses has shown to contain high salt concentration which could be inhibitory to anaerobic digestion [34]. Moreover, molasses contains some lethal compounds like benzoic acid, threonine and phenyl ethyl alcohol which inhibit methanogenesis [35, 36]. Several other factors like pH drop, increase in VFA concentration may cause inhibition [37, 38]. Therefore, due to these reasons, failure in the mono-digestion process of molasses negatively impacts the microbial community of the system.

In correspondence to mono-digestion, the Co-digestion process was more stable and suitable for digesting highly concentrated molasses with rice alcohol wastewater (RAW). This stability could be attributed to sharing essential micro and macronutrients in the co-substrates and dilute the toxic

chemicals in either of a substrate [11, 39]. As shown in Fig. 2a, during the acclimatization phase, the COD removal rate increased from 31.33 ± 0.5 to 52.00 ± 0.63% with an OLR value of 1.8 COD L^{-1}. d^{-1} in first 20 days. A sharp increase in COD removal rate (65.48 ± 0.82% to 90.72 ± 0.63%) was observed with a steady increase in OLR from 2.5 g COD L^{-1}. d^{-1} to 10 g COD L^{-1}. d^{-1}. When ORL was further increased from 10 to 16 g COD L^{-1}. d^{-1}, the COD removal rate remained constant (85.05 ± 1.19% – 90.25 ± 0.65%). However, pH was suddenly dropped when digesters were continuously fed with OLR 16 g COD L^{-1}. d^{-1}, as no upset was observed during the entire process of co-digestion as compared to mono-digestion. Thus, co-digestion was found to be a more stable process concerning the COD removal rate from molasses at a high loading rate. The co-digestion process greatly enhanced the digestion process and many comparative studies revealed that co-digesting multi-substrates confirms advantages over mono-digestion [40, 41]. Co-digestion stability was also confirmed by digesting pig manure and molasses alcohol wastewater by Shen et al. [11]. Many others have reported that co-digestion using animal manures enhances the performance of anaerobic digestion [42, 43, 44].

2.2. Methane Contents In Biogas Under Different Olr Conditions

In the co-digestion process, the percent of methane contents gradually increased (from 42–59%) with increasing OLR to 3.75 g COD L^{-1}. d^{-1} (Fig. 3a). However, when the OLR was increased to 7 g COD L^{-1}. d^{-1}, methane content suddenly dropped to 54.71% which could be attributed to its suppressive effect on the methanogens. Since it is obvious that increased OLR showed antagonistic effects on methanogenic communities [45]. With further increase in OLR, a gradual increase in methane content was observed which suggests that microbial communities get familiar to high load rate. The highest methane contents (64.47%) were seen at OLR 12 g COD L^{-1}. d^{-1}, and almost remained stable up to OLR 16 g COD L^{-1}. d^{-1}. In general, variation in the methane content in this study could probably be due to varying the concentrations of the OLR as consequences of its effect on the methanogens suppression. Similar to our results, De Vrieze et al. [46] obtained high methane content when molasses was digested with kitchen waste at an OLR value lower than 5 g COD L^{-1}.d^{-1}. Moreover, Mu et al. [38] reported a synergistic effect on methane production.

Methanogenic activity in co-digestion was found quite even than in mono-digestion. Figure 3b shows during acclimation phase I, methane contents produced in mono-digestion were quite comparable to co-digestion. This could be attributed to the addition of RAW with molasses in phase I. However, in phase II, when RAW was removed and only molasses was used as a substrate for digestion, a decrease in methane contents were observed by increasing OLR 9 g COD L^{-1}. d^{-1}. Similar observations were seen in the study of Eslami et al. [45]. Methane contents in biogas related to the balance of archaeal and bacterial community in the system, when OLR increased, the active bacterial community leads to the substantial accumulation of VFA. This increase in VFA contents sours the process and suppress the activity of archaeal community [47]. In phase III, again RAW and molasses were combined and OLR has

decreased to reacclimatize the process which produced the highest methane content (61.37%). Interestingly, in phase IV, when only molasses was subjected as a sole substrate up to 10 g COD L^{-1}. d^{-1}, the pH of the reactors started to decline along with methane contents. This two-time decrease in methane contents demonstrates that the digestion efficiency of molasses as a sole substrate depends on the inlet OLR. However, due to load shock in the influent OLR, the system causes low methane contents in the biogas produced. A similar phenomenon was reported by Meng et al. [12] in a two-stage reactor. The utilization of simple batch reactors in our study produced a higher amount of methane than Meng et al. [12] and is comparable to two-stage advanced reactors. This shows that methane can be produced successfully simply and cost-effectively. A decrease in methane content was also observed by De Vrieze et al. [10] while increasing OLR. Since it is evident from our study that compares to mono-digestion, co-digestion is an efficient way to digest high strength molasses with rice alcohol wastewater at OLR (16 g COD L^{-1}. d^{-1}) and achieve high methane contents 64.47%. The increased methane contents in the co-digestion process can be explained by an easily degradable fraction from the co-substrate, and well-established methanogens community could attribute to the increased production of methane.

2.3. Impact Of Increasing Olr On Ph

pH is one of the important parameters for evaluating the efficiency of an anaerobic digestion process. In the first 20 days, the acclimation period, high variation in pH values were observed in both the mono-digestion and co-digestion process. The COD removal rate and methane contents were also gradually increased during this time. After the acclimation stage, the pH remained stable throughout the co-digestion process regardless of the continuous increase in OLR. However, in the mono-digestion process, pH dropped twice, particularly when the OLR reached to 9 &10 g COD $L^{-1}d^{-1}$. This drop-in pH in mono-digestion led to the process of instability. The stability in pH of co-digestion suggests that the process is highly buffered [38]. De Vreize et al. [10] reported that digesting molasses as a single substrate at high OLR can cause a drop in pH, probably due to high salt contents in molasses or deficiency of some important micronutrients.

2.4. Detection Of Vfas Concentration

After hydrolysis and acidogenic phase in anaerobic digestion, the produced VFAs are consumed by methanogens and produce methane and CO2. Total VFA concentrations are the key parameters to estimate the efficiency of the digestion process. Volatile fatty acid (VFA) concentrations are shown in Fig. 4a. Initially, total VFA accumulation in both the processes was observed in the range of 3584.75 mg/L to 4653.60 mg/L. Rapid VFA accumulation could be due to unbalanced acidogenic, acetogenic and methanogenic communities [48]. Within the total VFA content, propionic acid and acetic acid were in the highest concentration followed by Isobutyric acid (Fig. 4b, c). This was associated with the pH fluctuation in the initial stage (acclimation stage) of both processes. However, this did not disturb the process; probably both processes started with co-substrate with low OLR 1.87 g COD L^{-1}. d^{-1}. The

other possible reason could be the increase in buffering capacity due to co-substrate [49, 50]. After acclimation stage (from day 28 to 56), the process gets stabilized and the pH becomes neutral, the total VFA concentration in Phase I of mono-digestion was ranged from 159.88 mg/L to 310.81 mg/L, while in co-digestion process, a relatively high concentration of VFA (675.93 to 1633.12 mg/L) was observed. During the entire co-digestion process, total VFA concentrations never reached the limit that led to failing the process. However, the mono-digestion process failed two times when the total VFA exceed from 2059.66 mg/L at day 84 and 1896.9 mg/L on the last day (156) of the process. This suggested that total VFA concentration exceeding the above limits could be an alarming situation for process failure. While at these two inhibitory stages, acetic acid, propionic acid, and Isobutyric acid were found in their highest concentrations (Fig. 4b, c) illustrating these could be the possible reason for process failure.

Among these acids, propionic acid was produced in high concentrations in both processes could be due to the presence of a high abundance of *Propionibacteriaceae* in bacterial communities (Fig. 5a). Propionic acid accumulation has already been reported in the literature for its inhibitory effects, that its higher oxidation is unfavorable for the smooth operation of the AD process. Furthermore, various factors such as pH, temperature, hydrogen partial pressure, and other VFAs accelerates propionic acid oxidation [51, 16, 52]. As a result, it inhibits methanogenic bacteria that utilize acetate for methane production [53]. Lee et al. [54] also reported that the accumulation of propionic acid could decrease process performance and lead to process failure. Our results are in agreement with Lee et al. [55] who stated that an alarming concentration of propionic acid is 1000–2000 mg/L and suggested that exceeding this limit can inhibit methanogens. In our study, we confirmed that using molasses as a sole substrate in anaerobic digestion, monitoring of propionic acid and total VFA should be determined periodically.

2.4. Microbial Community

2.4.1. Sequencing and microbial community in response to increasing OLRs

A total of 1,677,506 paired reads for the bacterial community was obtained, which were clustered into OTUs based on ≤ 97% similarity. OTU numbers detected in each sample were in the range of 378 to 494. On the other hand, archaeal community reads were detected in 1,651,713 pairs and after clustering into OTU on basis of ≤ 97% similarity, the detected OTUs in all samples were ranged from 94 to 84. After a continuous increase in the OLR clear difference in OTU numbers was observed in each sample of both digestion processes.

2.5.1.1. Composition Of Bacterial Community

The composition of the bacterial community expressed at family level classification showed a marked difference in their structure in both mono-digestion and co-digestion processes and during each particular

process (Fig. 5a). As mentioned earlier both the process was started with the same inoculum, the majority of microbes observed in MR15 (day 0) was dominated by *Propionibactereaceae* (24.13%) followed by *anaerolinaceae* (14.42%), *streptococaceae* (14.05%), *Bacteriodetes_vadinHA17* (9.91%) and *Bogoriellaceae* (4.57%). When the digestion process continued, variations in the community were observed in MR35 and M35 at the influent rate of OLR 35 g COD L−1d−1 in both processes. During this time, in MR35, an unexpected increase in abundance of *Beijerinckiaceae* from class *alpha-proteobacteria* and other *uncultured bacterium* was observed by suppressing the abundance of other families. Hameed et al. [55] and Zhang et al. [56] identified the genera *beijerinckia* and other related bacteria from the same family in anaerobic communities with stable acetoclastic methanogenesis (74–84%) that shows syntrophic association with methanogens. The bacteria related to family *Beijerinkia* are known for nitrogen reduction in the anaerobic system [56]. However, in other samples, *Beijerinckiaceae* and *uncultured-bacterium* did not exceed 0.02%. In contrast to MR35, the composition of bacterial communities was quite stable in M35 when compared with day 0 (MR15). A relative decrease was observed in *Propionibacteriaceae, Streptococaceae* and *Bacteriodetes_vadinHA17* with the increase in abundance of *Anaerolinaceae*, *Eubacteriacea* and *Bogoriellaceae* (Fig. 5a). These shifts in increase and decrease in the abundance of specific families can be correlated with the performance of reactors. As can be seen in Figs. 2 & 3, low COD removal rate and methane contents produced in an early stage of operation. Additionally, the fluctuation in pH and high concentration of propionic acid could be attributed to the presence of *Propionibacteriaceae* in high abundance in both systems. In M35 and MR35 a remarkable high COD removal rate, methane contents and low concentration of propionic acid were observed. However, at these stages, the decrease in *Propionibacteriaceae* abundance was seen. At the last stages of both mono-digestion (M51) and co-digestion (MR75), a great difference in microbial compositions were seen. In the mono-digestion process, a two-time increase in abundance of *Propionibacteriaceae* (31.35%), *Carnobacteriaceae* (6.92%) and *Eubacteriaceae* (8.07%) by suppressing other families could be the possible reason for a process upset. In comparison to M51, the compositional abundance in co-digestion (MR75) was observed as *Propionobacteriaceae*, *Anaerolinaceae*, *Carnobacteriaceae*, *Eubacteriaceae, Bogorielaceae* with their relative abundances, 28.22%, 10.96%, 23.69%, 11.62%, and 7.80% respectively are in association with good performance of the process. Besides these, some other bacteria were also observed which had an impact on biodegradation.

The relative proportion of bacterial communities responsible for hydrolytic, acidogenic and acetogenic phases in the digestion process should be co-existed with each other for stable performance. This can be associated with loading concentration of the influent which resulted in an increase or decrease in abundance of specific bacterial community and led the process prone to disturbance, as can be seen from the process upsets in the mono-digestion process, dynamic changes occurred in bacterial composition.

2.5.1.2. Composition Of Archaeal Community

The distribution of the archaeal community is shown in Fig. 5b. During both digestion processes, the composition and abundance of methanogens varied upon OLRs concentrations. Among all methanogens, *Methanosaetaceae* and *Methanobacteriaceae* were dominant in all stages. Methanosaetaceae and *Methanosarcinaceae* were increased when the process was stabilized at the stage of M35 and MR35, which probably decreased *Methanobacteriaceae*. At this stage, in the mono-digestion and co-digestion process, methane content in biogas was higher than 55%. This was also accompanied by a good COD removal rate which suggested a good performance of both processes.

On the other hand, in the mono-digestion process in stage M51, a decrease in all major methanogens and process performance was observed. This was associated with the increase of *Propionibacteriaceae* (Fig. 5a) in the digester producing propionic acid (Fig. 3c) and led to the suppression of methanogens (Fig. 5b). In comparison to mono-digestion, stability in all major methanogens i.e *Methanosaetaceae*, *Methanobacteriaceae*, *Methanosarcinaceae* and *Methanom icrobiales* were found in all stages of co-digestion, with some ups and downs. In general, the whole process performance of co-digestion was better than mono-digestion. *Methanosaetaceae*, the methanogens can survive at both high and low concentrations of acetate [57, 58]. In both the mono-digestion and co-digestion process, *Methanobacteriaceae* was found in all stages and ranged from 12.02–20.44%, which is in the line with the work of Gagliano et al. [59] and Ziganshin et al. [60]. *Methanobacteriaceae* is the primary user of formate and H2/CO2 substrates for methane formation. This suggested the co-existence of acetoclastic and hydrogenotrophic methanogenesis in both processes. The same phenomena were also reported by Lu et al. [17] and Wang et al. [14]. In the co-digestion process, *Methanosarcinaceae* was found as the second most abundant group after *Methanosaetaceae*. The members of *Methanosarcinaceae* were acetoclastic and methanotrophic methanogens and were capable of using acetic acid, CO2, methanol, methylamine as a substrate for methane formation similar to *Methanosaetaceae*. The high concentration of *Methanosarcinaceae* and *Methanobacteriaceae* in the last two stages of co-digestion confirmed the production of high methane contents.

3. Conclusion

Molasses is a byproduct of the sugarcane industry and is one of the attractive sources for biogas production due to its high organic fraction. However, due to high density and salinity, its anaerobic digestion is very difficult. The current study investigated the digestion of molasses in both mono-digestion and co-digestion process. Co-digestion was found as the most sophisticated and stable process than mono-digestion. In the co-digestion process, highly concentrated molasses can easily be digested than the mono-digestion process. The influent COD in co-digestion reached to its maximum value of 80 g L^{-1} without any disturbance, while in mono-digestion process, two times disturbance occurred when the influent rate reached to 50 g L^{-1}. This disturbance in the mono-digestion process was due to the increased accumulation of total VFAs that decreases the COD removal rate and methane contents of biogas. Microbial community composition in the co-digestion process indicated the relative abundance of hydrolytic and acidogenic bacteria which were syntrophically coexisted with methanogens

rather than in mono-digestion. Overall, co-digestion could potentially be a promising way to digest high strength molasses at high OLR.

4. Materials And Methods

4.1. Sludge and waste water

The anaerobic sludge was obtained from IC reactor treating bagasse spray wastewater at Guangxi Guitang (Group) Co. Ltd., while cane molasses and rice alcohol wastewater (RAW) were also obtained from the same company.

4.2. Digesters Setup

Plastic containers with the total volume of 5L and working volume 4L were used as digester tanks which were sealed with silicon and a rubber stopper. In each container, three ports were made for affluent, influents and biogas collection. Airtightness of each digester was safeguarded to avoid any leakage (Fig. 1).

4.3. Operating Conditions For Co-digestion And Mono-digestion Experiment

In the co-digestion process of molasses, RAW was used as a co-substrate. Initial OLR was 1.8 g COD $L^{-1}d^{-1}$ at a 1:1 ratio. After acclimatization, the concentration of molasses was increased gradually, while RAW concentration was kept constant as shown in Table 1. OLR was also increased at regular intervals when 80 percent of the COD removal rate was achieved.

Parallel to the co-digestion process, molasses was digested alone (mono-digestion process) in the second set of experiment. The process was divided into 4 phases (Table 2). In phase I, RAW was co-digested with molasses (from OLR 1.8 to 7 g COD $L^{-1}d^{-1}$). In phase II, molasses was fed to digesters without co-substrate at OLR 6.8 g COD $L^{-1}d^{-1}$, and ended at day 83 when OLR reached to 9 g COD $L^{-1}d^{-1}$. At this time, the efficiency of the digester decreased due to instability in pH values. In phase III, OLR was then decreased to 4 g COD $L^{-1}d^{-1}$ and a co-substrate (RAW) was used again to acclimatize the process with a periodic increase in OLR to 7 g COD $L^{-1}d^{-1}$. In phase IV, molasses was digested alone at an increasing rate of OLR from 6 g COD $L^{-1}d^{-1}$ to maximum OLR 10 g COD $L^{-1}d^{-1}$.

Each set of experiments was run in triplicate at 35 ± 2ºC. In the beginning, the HRT of each experiment was 9 days. On the maximum COD removal rate, the HRT was decreased to 5 days and kept constant throughout the experiment. The pH was measured daily with the exchange of wastewater, while COD and

percent methane contents were examined at each 3 days interval. Samples for VFA contents were collected every week and stored at − 20°C for further analysis.

Table 1
Strategy for co-digestion of molasses and RAW

S. No.	Run time (Days)	Influent COD(g/L)	OLR (g COD/L.d)	HRT (Days)	Flow rate (L/d)	M:RAW
1	0–20	15	1.875	8	0.5	1:1
2	21–29	20	2.5	8	0.5	1:1
3	30–38	25	3.12	8	0.5	2:1
4	39–47	30	6	5	0.8	3:1
5	48–56	35	7	5	0.8	4:1
6	57–65	40	8	5	0.8	5:1
7	66–74	45	9	5	0.8	5:1
8	75–83	50	10	5	0.8	5:1
9	84–92	55	11	5	0.8	5:1
10	93–101	60	12	5	0.8	5:1
11	102–111	65	13	5	0.8	6:1
12	112–120	70	14	5	0.8	6:1
13	121–129	75	15	5	0.8	7:1
14	130–147	80	16	5	0.8	8:1

Table 2
Mono-digestion of molasses

Operating conditions during digestion of molasses (Experiment begins with Co-digestion)						
S.No	Run time (Days)	influent COD (g/L)	OLR (g COD/L.d)	HRT (Days)	Flow rate (L/d)	M:RAW
1	0–20	15	1.875	8	0.5	1:1
2	21–29	20	2.5	8	0.5	1:1
3	30–38	25	3.12	8	0.5	2:1
4	39–47	30	6	5	0.8	3:1
5	48–56	35	7	5	0.8	4:1
Remove RAW and feed only molasses						
6	57–65	35	7	5	0.8	1:00
7	66–74	40	8	5	0.8	1:00
8	75–83	45	9	5	0.8	1:00
Acclimitization of reactors with co-digestion of molasses and RAW						
9	84–88	20	4	5	0.8	3:1
10	89–98	25	5	5	0.8	4:1
11	99–103	30	6	5	0.8	4:1
12	104–108	35	7	5	0.8	4:1
Remove RAW and feed only molasses						
13	109–117	30	6	5	0.8	1:00
14	118–126	35	7	5	0.8	1:00
15	127–135	40	8	5	0.8	1:00
16	136–144	45	9	5	0.8	1:00
17	145–153	50	10	5	0.8	1:00

4.4. Analytical Methods

The pH of the effluent and influent was measured daily using Lichen Model pH-100 meter. COD concentration was determined using HACH (DRB 200 (COD 200-15000)). Biogas produced by each digester was collected in biogas bags and analyzed by gas chromatography, Shimadzu GC-14C

(Shimadzu Co. Ltd., Japan) using a Porapak Q (80–100 mesh; 0.3 mm × 2 m) capillary column equipped with FID detector. Operating temperatures for inlet, column, and detector were set at 100 ºC, 60 ºC, and 100 ºC respectively. The hydrogen gas, nitrogen as a carrier gas and oxygen pressures were established to 40 Kpa, 65 Kpa, and 30 Kpa, respectively. The gas sample was injected manually with a total volume of 3 µL. Methane concentration was calculated by comparing the peak area resulted from the standard sample (99.9% methane).

Volatile fatty acid (VFA) concentrations were measured by Shimadzu 2010 gas chromatograph (Shimadzu Co. Ltd., Japan) using FID and a capillary column type SGE BP 21. Helium was used as a carrier gas at a flow rate of 190.4 ml min^{-1}, with a split ratio of 100 giving a flow rate of 1.86 ml^{-1} in the column and a 3.0 ml min^{-1} purge. The GC oven temperature was programmed to increase from 60 to 210 ºC in 15 min, with a final hold time of 3 min. Temperatures of injector and detector were 200 and 250 ºC respectively. Samples were acidified using 3% formic acid. Three standard solutions containing 50, 250 and 500 mg L^{-1} of acetic acid, propionic acid, isobutyric acid, butyric, isovaleric, valeric acids were used for VFA calibration.

4.5. High-throughput Sequencing For Microbial Community

Sludge samples were collected for the microbial community at 4 different periods from the early stage to the last stage of digestion. Before sample collection, each digester was shaken to homogenize the sludge content with the fermenting substrate. About 50 g of sample was taken and part of it was stored at –80oC for further analysis. The samples were named M35, M50, and M51 for the mono-digestion process while MR35, MR50, and MR75 were labeled for the co-digestion process. Besides these, a 0-day sample labeled as MR15 was taken from sludge as the same sludge was used for both processes.

4.5.1 Extraction Of Dna, Pcr Amplification And Sequencing

Fast DNA_ Spin Kit for Soil (Q-BIOgene, Carlsbad, USA) was used to extract genomic DNA from the samples according to the manufacturer’s instructions. V3-V4 regions were amplified was targeted and primers 5'-ACTCCTACGGGAGGCAGCA-3' and 5'- GGACTACHVGGGTWTCTAAT-3' for bacteria Arch349F: 5'-GYGCASCAGKCGMGAAW-3' and Arch806R: 5'-GGACTACVSGGGTATCTAAT-3' for methanogens (Lu et al., 2019). PCR amplification was applied according to Shen et al (2014). Constituents taken for PCR analysis were 10 × PCR buffer, 0.25 mmol/L deoxyribonucleotide triphosphate dNTPs, take each primer concentration 0.4 µmol/L, 1U Ex Taq polymerase (TaKaRa Company, Japan) and 10 ng of sample DNA template. All of these were added to molecular grade water to make a total volume of 25 µL. Conditional steps for operating PCR were as follows: after pre-denaturation step at 94º for 5 min, 25 cycles were performed in three steps: denaturation at 94ºC for 1 min, annealing temperature 65ºC for 1 min decreased to 56ºC by decreasing 0.5ºC per cycle and an extension step at 72ºC for 1 min, again followed by 10 cycles at annealing temperature 55ºC and finally followed by an extension for 8 min at 72ºC. After

the amplification process, agarose gel electrophoresis was performed to verify and quantify the concentration of PCR products. After the recovery and purification of PCR products to work as a template, a reconditioning PCR was performed as described by Shen et al [11] to remove false-positive results and to obtain highly specific products. The PCR products were then sequenced using High throughput sequencing, and the generated sequences were used to identify specific operational taxonomic units (OTUs) for bacterial and methanogen taxonomy and their community comparisons at the family level.

Declarations

Ethics approval and consent to participate

(Not applicable)

Consent for publication

(Not applicable)

Availability of data and materials

(Not applicable)

Declaration of interests

 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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Not Applicable

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Authors contributions

Sohail Khan: Methodology, Investigation, Data Curation, Writing - Original Draft Conceptualization, **Qiong Jiang**: Formal analysis **Fuzhi Lu, Chengjian Jiang, Muhammad Kashif** Review & Editing, **Peihong Shen**:

Supervision, Resources, Project administration, Visualization

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Figures

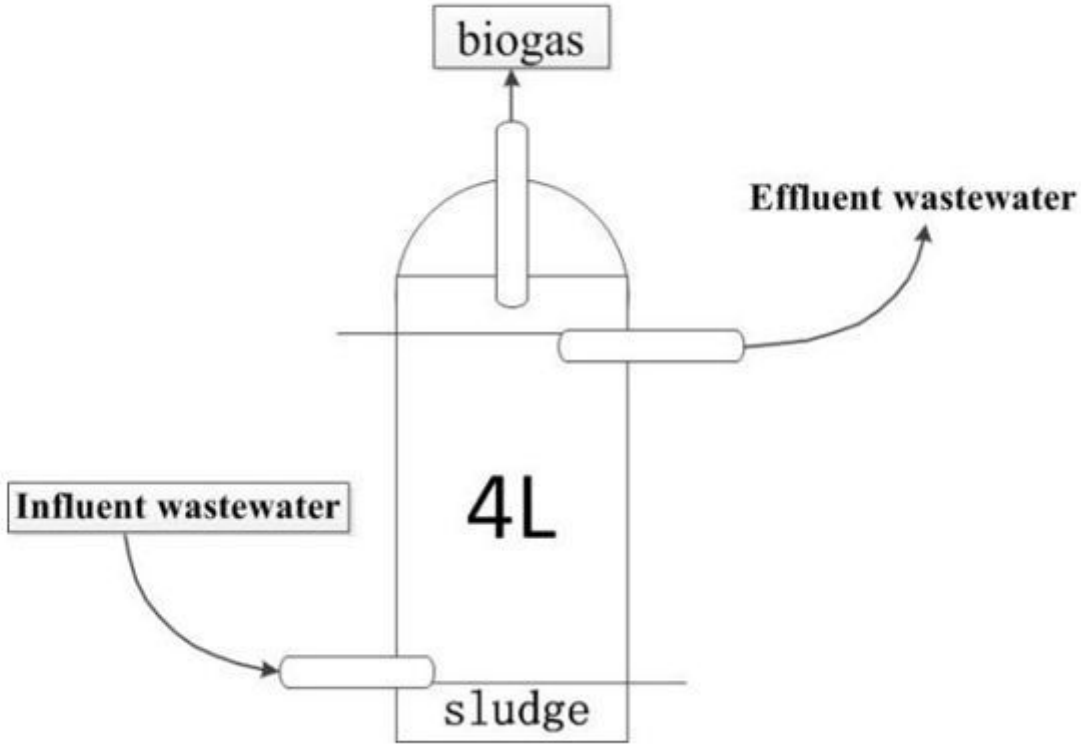

Figure 1

A schematic diagram of anaerobic batch reactor

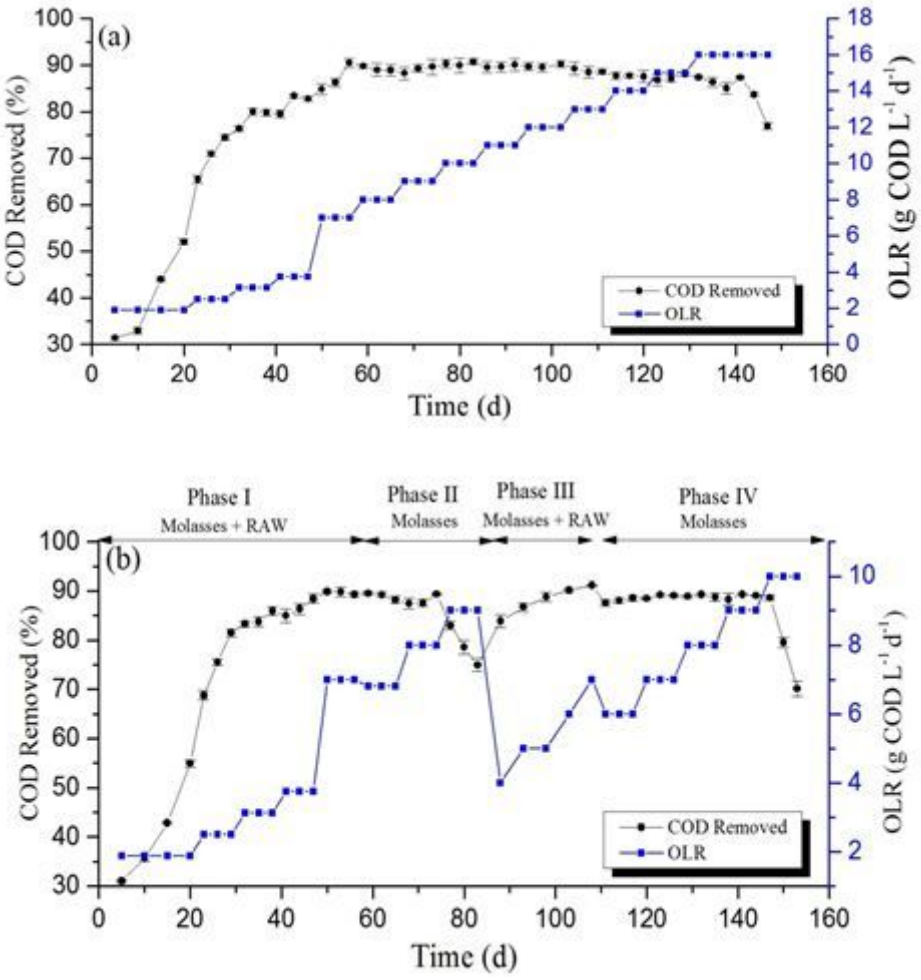

Figure 2

(a) Percent COD removal rate at increasing OLR in co-digestion process. (b) Percent COD removal rate at increasing OLR in mono-digestion process. Phase 1 show acclimatization of the digestion process by co-digestion of molasses and RAW. Phase 2 show mono-digestions of only molasses. Phase 3 showing co-digestion to acclimatize the reaction, and Phase 4 showing only molasses digestion to evaluate the highest OLR of only Molasses

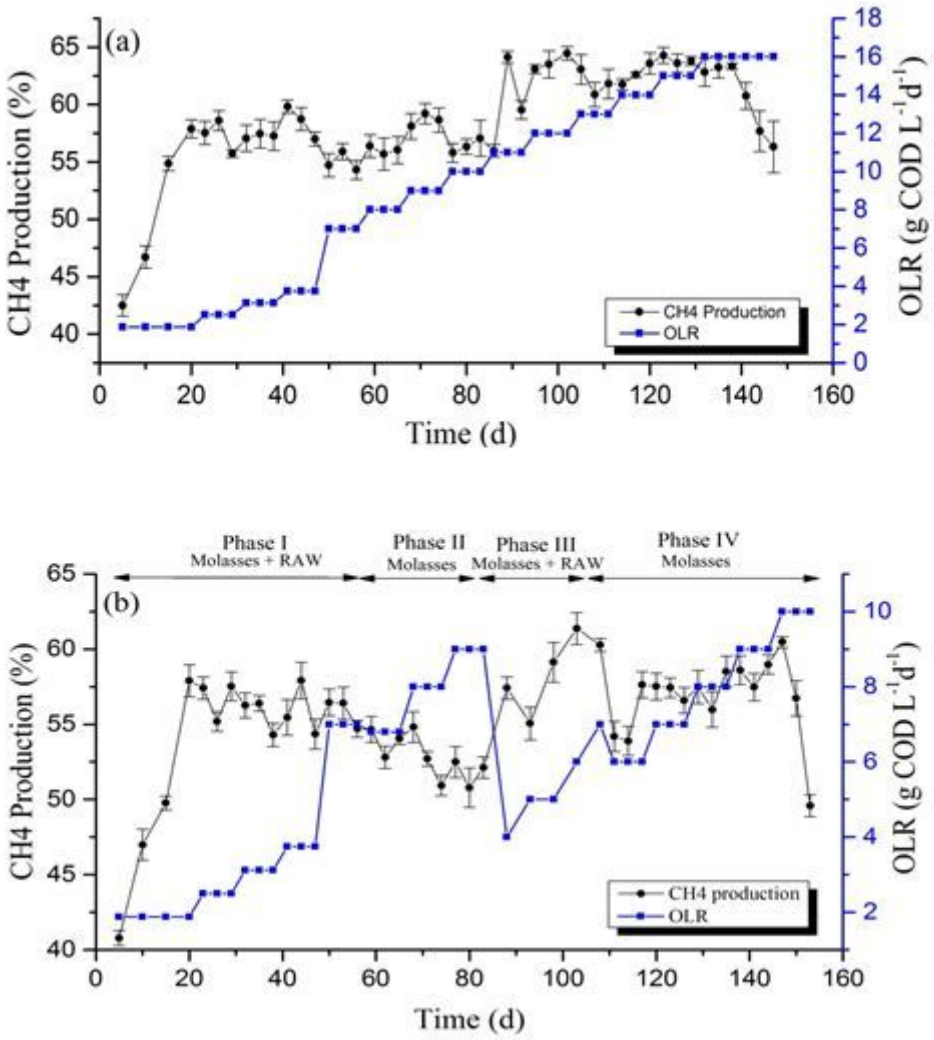

Figure 3

(a) Percent methane contents at increasing OLR in co-digestion process. (b) Percent methane contents at increasing OLR in mono-digestion process. Phase I show acclimatization of the digestion process by co-digestion of Molasses and RAW. Phase II show mono-digestions of only Molasses. Phase III showing co-digestion to acclimatize the reaction again, While Phase IV showing only Molasses Digestion to evaluate the highest OLR of Only Molasses

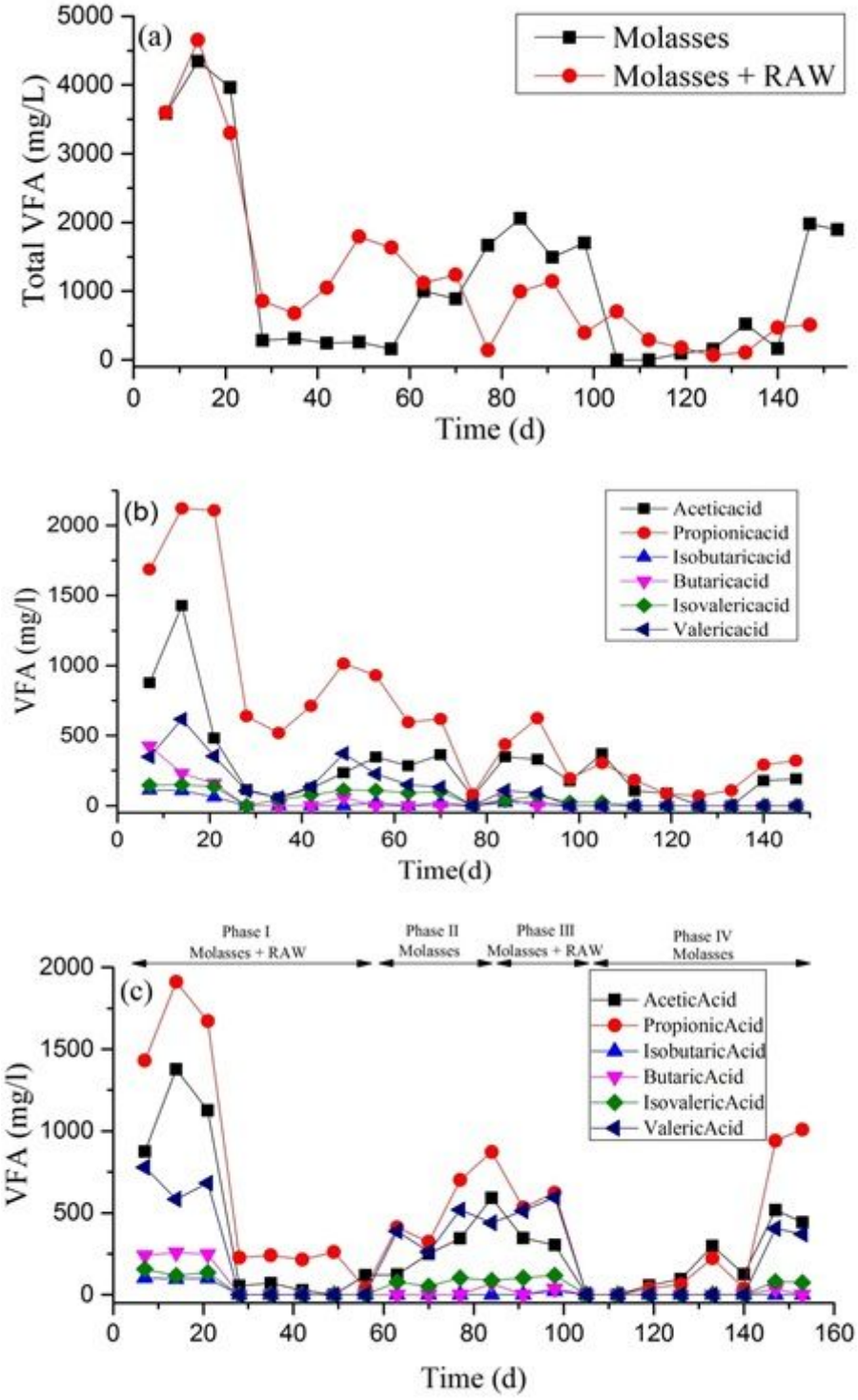

Figure 4

(a) Total VFA concentration in both processes. (b) VFAs accumulated in co-digestion process. (c) VFAs produced in mono-digestion process.

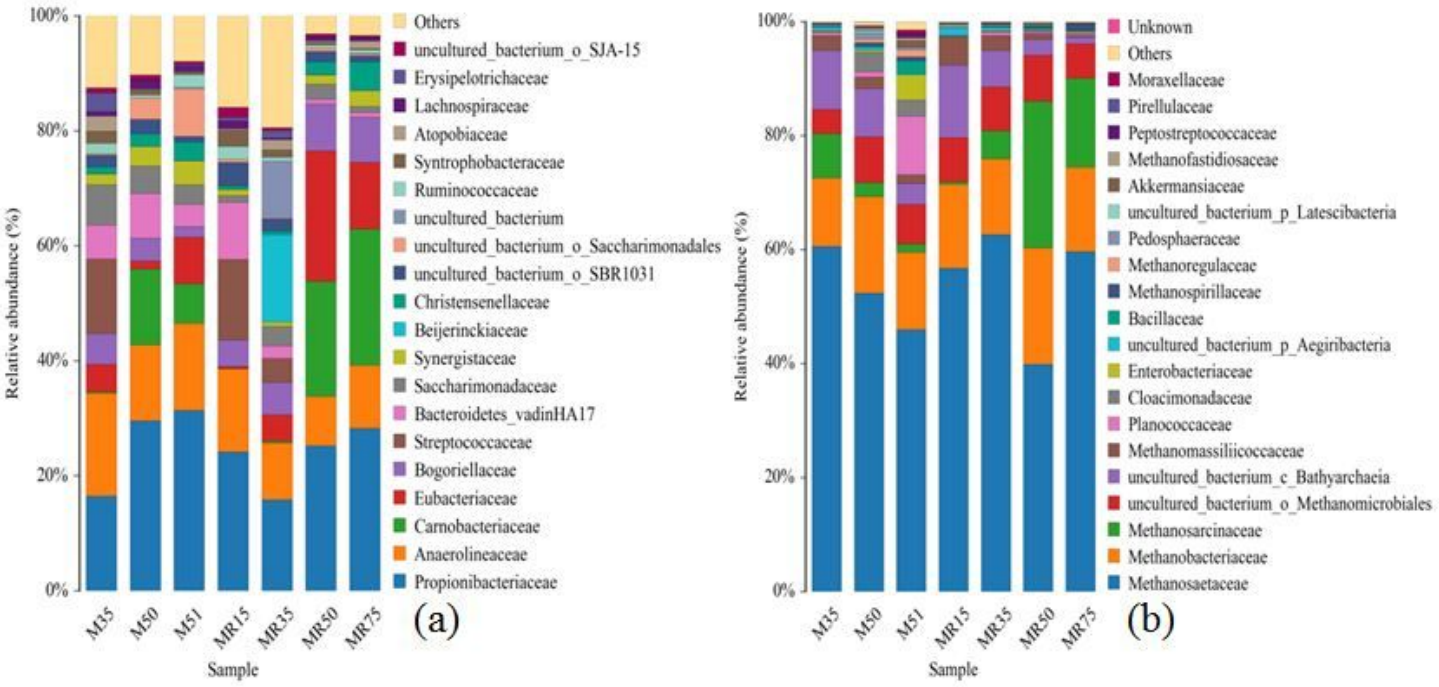

Figure 5

Changes in relative abundance of (a) Bacterial communities and (b) Archaeal communities in mono and co-digestion at different stages. The corresponding samples M denote mono-digestion (M35, M50 and M51) and MR denotes co-digestion (MR 35, MR50 and MR75). The sample MR15 was taken at 0 day indicates the startup inoculum for both processes.