Two- vs. single-stage anaerobic reactors: evaluation of effluent quality and energy production potential using sucrose-based wastewater

V. T. Mota and M. Zaiat

ABSTRACT

Two- and single-stage anaerobic treatment systems were assessed for treatment performance and for bioenergy production from sucrose-based wastewater. In the two-stage system, a hydrogen-producing upflow anaerobic sludge blanket reactor (HU reactor) was used in the acidogenic phase. The methanogenic reactor of the two-stage system (MF reactor) and the single-stage reactor (SSF reactor) were structured fixed-bed reactors. The two-stage system showed superior performance, evidenced by lower organic acids, chemical oxygen demand (COD) and suspended solids concentrations in the effluent, and higher biogas methane content and yield. Continuous and stable H_2 production was obtained in the acidogenic reactor. At the end of operation, the organic loading rates applied to the two- and single-stage systems were 6.4 and 5.2 gCOD L^{-1} d^{-1}, respectively. Under these conditions, the effluent soluble COD and volatile suspended solids (VSS) concentrations were 165 and 92 mg L^{-1} in the two-stage system, and 256 and 244 mg L^{-1} in the single-stage system, respectively. The energy yield of the two-stage system was 20.69 kJ $g^{-1}COD_{added}$, which was 34% higher than the yield of the single-stage system. The sequencing analyses showed that the archaeal distribution changed little between the inoculum and sludge from the MF reactor, in which acetoclastic *Methanosaeta* was predominant. However, hydrogenotrophic *Methanospirillum* was found most, followed by *Methanosaeta*, in the sludge from the SSF reactor.

Key words | bioenergy, hydrogen, methane, pH, structured fixed-bed reactor, two-stage anaerobic treatment

V. T. Mota (corresponding author)
M. Zaiat
Biological Processes Laboratory (LPB), Department of Hydraulics and Sanitary Engineering (SHS), São Carlos School of Engineering (EESC), University of São Paulo (USP), Avenida João Dagnone, 1100, São Carlos, São Paulo 13563-120, Brazil
E-mail: *vtaina@hotmail.com*; *vtaina@usp.br*

INTRODUCTION

Anaerobic reactors are widely used in the treatment of domestic and industrial wastewater. In addition to being relatively simple and inexpensive for removing pollutants, especially biodegradable organic matter, energy can be obtained by recovering the methane produced. However, under stressing conditions, such as shock loading and toxic materials input, disequilibrium can occur due to kinetic and thermodynamic limitations, especially of methanogens. One possible way to improve the performance of anaerobic digestion uses the two-stage anaerobic system. This consists of two reactors placed in series, wherein acidogenesis and methanogenesis prevail in the first and second reactors, respectively, because of the selective pressure. Through the identification and optimization of the limiting steps, this configuration enables the maintenance of more favourable conditions for distinct microbial groups (Ke *et al.* 2005). It also avoids the imbalance between organic acid production by acidogenic bacteria, that have faster growth-rates, and organic acid consumption by the methanogenic archaea, that are more sensitive to environmental conditions (pH, toxics, temperature).

Ghosh *et al.* (1985) evaluated the performance of two-stage anaerobic reactors, laboratory, pilot and full scale, used in treating various types of industrial effluents, and compared the performance to that of single-stage anaerobic reactors. The most significant advantages found by Ghosh *et al.* (1985) and by others, of two-stage anaerobic systems applied to the treatment of liquid effluents, are: the possibility of operating with higher organic loadings (Cohen *et al.* 1982; Cho 1983); increased calorific value of biogas, that is, higher methane content (Yeoh 1997); higher chemical oxygen demand (COD) removal efficiency (Azbar & Speece 2001;

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Ferraz Júnior *et al.* 2016); and higher methane yield (Yeoh 1997; Nasr *et al.* 2012; Lullio *et al.* 2014). Despite the several advantages, there are relatively few reports of the use of two-stage anaerobic reactors in full-scale wastewater treatment systems (Ghosh *et al.* 1985; Young *et al.* 2000; van Groenestijn *et al.* 2006; Perendeci *et al.* 2012; Ma *et al.* 2017). The lack of experience with the process results in difficulties in design and construction (Ke *et al.* 2005; Rapport *et al.* 2008).

Innovation in reactor design is also related to improving the efficiency of anaerobic biological reactors. The configuration of a continuous anaerobic structured fixed-bed reactor (AnSFBR) is very attractive due to its low operating requirements and applicability to small wastewater treatment plants. The description of this configuration was first published internationally by Picanço *et al.* (2001). Since then, it has been applied in bench-scale studies (Mockaitis *et al.* 2014; Fuess *et al.* 2017). AnSFBR design has some advantages with respect to randomly packed fixed-bed, expanded-bed and fluidized-bed reactors. These advantages include the prevention of solid accumulation as well as clogging and channelling effects, higher biomass retention capacity (Mockaitis *et al.* 2014), and lower energy input (Fuess *et al.* 2017). Fuess *et al.* (2017) also described better overall performance of an AnSFBR, compared to an upflow anaerobic sludge blanket (UASB) reactor, applied to the thermophilic treatment of acidified vinasse.

Most of the studies that compared similar methanogenic reactors fed with raw and acidified wastewater, had been performed using UASB (Kim *et al.* 2004; Ferraz Júnior *et al.* 2016) and stirred reactors (Yeoh 1997; Azbar & Speece 2001; Nasr *et al.* 2012). However, there is a lack of studies on the effect of applying acidogenic reactors prior to fixed-bed methanogenic reactors. Considering that both two-stage anaerobic digestion and AnSFBR are promising and sustainable for the improvement of wastewater treatment, this study aimed to compare single- and two-stage systems, applying AnSFBR for methanogenesis. A completely biodegradable synthetic medium based on sucrose was used, due to the emerging status of the full-scale application. This allowed better understanding of the process dynamics.

MATERIAL AND METHODS

Reactor configurations

The reactors were cylindric and made of acrylic. The two-stage system consisted of a UASB reactor with 2.2 L working volume and 6.3 cm internal diameter, used for acidogenesis; this was followed by a structured fixed-bed reactor with 3.8 L working volume and 7.9 cm internal diameter, used for methanogenesis. The single-stage system consisted of a structured fixed-bed reactor with 6.4 L working volume and 9.6 cm internal diameter. The middle compartments of the structured fixed-bed reactors were filled with polyurethane foam strips (transversal area of 2 cm × 2 cm), which were orderly placed vertically, and fixed in the extremities to metal plates. The bed porosity was approximately 90% (Figure 1). Similar configuration is shown in Mockaitis *et al.* (2014). The choice of the support material is based on Picanço *et al.* (2001), who found that, in structured fixed-bed reactors, polyurethane foam and special ceramic had better biomass adhesion capacity than polyvinyl chloride and refractory brick.

The reactors were named as follows: (i) HU reactor: hydrogenogenic/acidogenic UASB reactor, used for acidogenesis in the two-stage system; (ii) MF reactor: methanogenic structured fixed-bed reactor, used for methanogenesis in the two-stage system; (iii) SSF reactor: single-stage structured fixed-bed reactor, used for acidogenesis coupled to methanogenesis in the single-stage system.

The hydrodynamics of the reactors were assessed using step stimulus-response tests, according to Levenspiel (1999). A saline solution with an initial concentration of 10 g NaCl L^{-1} was introduced continuously into the reactors (previously filled with clean water) at a flow rate corresponding to the hydraulic retention times (HRT) evaluated.

Substrate

The HU and SSF reactors were fed with sucrose-based wastewater containing demerara sugar (Native®) and a nutrient solution, in the following concentrations (mg L^{-1}): NH_4Cl (170), $CaCl_2{\cdot}2H_2O$ (8), KH_2PO_4 (37), $MgSO_4{\cdot}4H_2O$ (9), $FeCl_3{\cdot}4H_2O$ (2), $CoCl_2{\cdot}6H_2O$ (2), $MnCl_2{\cdot}4H_2O$ (0.5), $CuCl_2{\cdot}2H_2O$ (0.03), $ZnCl_2$ (0.05), H_3BO_3 (0.05), $(NH4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.09), $Na_2SeO_3{\cdot}5H_2O$ (0.1), $NiCl_2{\cdot}6H_2O$ (0.05), EDTA (1), HCl 36% (1 μL L^{-1}). In the raw influent of the HU reactor, the concentration of demerara sugar averaged approximately 4.8 gCOD L^{-1} and no buffer agent was added. Details of the HU operating conditions are given in Mota *et al.* (2018), in which this reactor is named the UF-2 reactor. The MF reactor was fed with acidified wastewater from the HU reactor.

During the acclimation period, which lasted 38 days, the methanogenic reactors (MF and SSF reactors) were fed with diluted wastewater. From days 0 to 17 (acclimation 1), the feeding was diluted 5-fold. From days 18 to 38

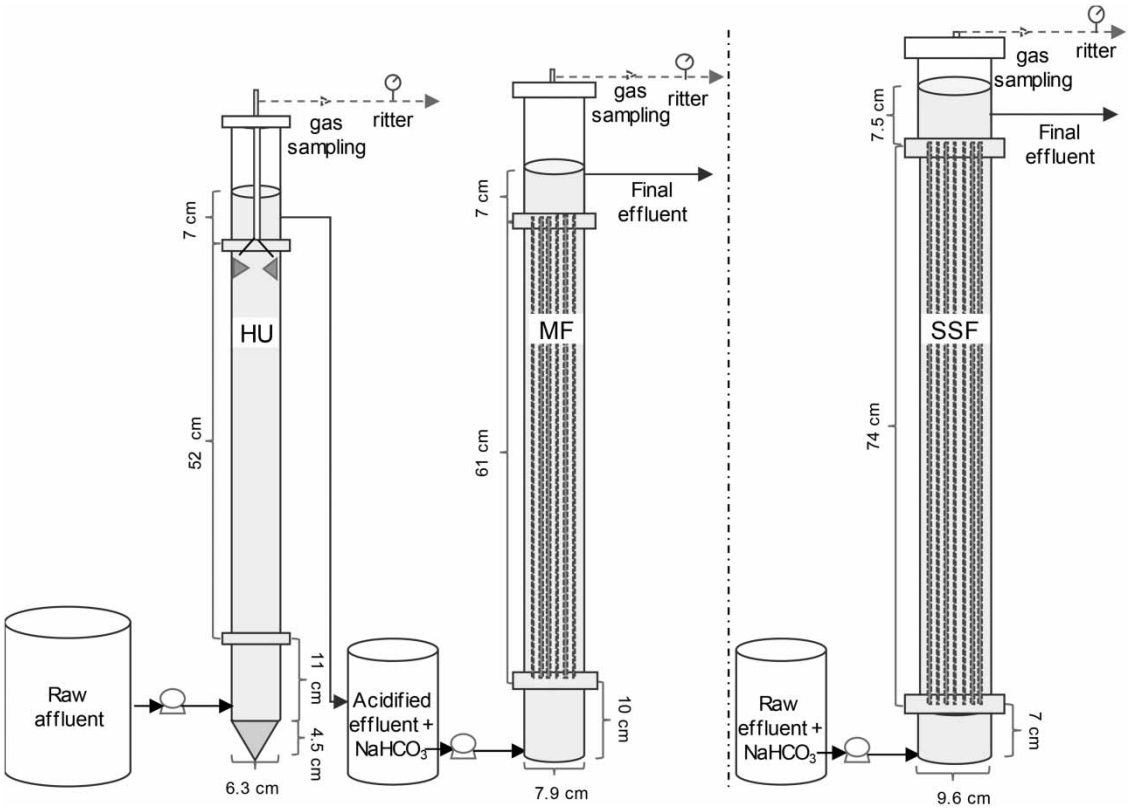

Figure 1 | Schematic diagram of the two-stage (left) and the single-stage (right) systems. HU reactor, MF reactor, SSF reactor.

(acclimation 2), the feeding was diluted 3.3-fold. $NaHCO_3$ was added as a buffer agent, to the feeding of the MF and SSF reactors, at concentrations of $1\ g\ L^{-1}$ and $1.5\ g\ L^{-1}$ during acclimation periods 1 and 2. Following acclimation, the MF reactor was fed with acidified effluent without dilution (averaging $4.4\ gCOD\ L^{-1}$), and the SSF reactor was fed with raw wastewater (averaging $4.8\ gCOD\ L^{-1}$). The $NaHCO_3$ was added to the influents of the MF and SSF reactors at concentrations of $5\ g\ L^{-1}$ from days 39 to 73, and of $4.16\ g\ L^{-1}$ from days 74 to 178. Feeding of the MF and SSF reactors was also supplemented with yeast extract at a concentration of $200\ mg\ L^{-1}$. It was diluted 5- and 3.3-fold (40 and $61\ mg\ L^{-1}$), respectively, during acclimation periods 1 and 2.

Inoculation and operating conditions

The reactors were inoculated with sludge from UASB reactors, used to treat slaughterhouse wastewater. The HU reactor was inoculated with sludge from a plant located in Pereiras (SP, Brazil), and the MF and SSF reactors were inoculated with sludge from a plant located in Tietê (SP, Brazil). The granules were completely crushed with a blender before inoculation. This procedure was adopted to suspend the cells and allow the colonization of the inner layers of the support material and biofilm formation on the foam surface. Following inoculation, the reactors were filled with their respective wastewaters, and left standing for 24 h before starting continuous feeding. The initial sludge concentration in the reactors was $15\ gVTS\ L^{-1}$. The temperature was maintained at $30 \pm 2\ ^{\circ}C$.

The operation was divided into two periods of acclimation, described in section 'Substrate', and into seven phases of operation. The flow rates of the MF and SSF reactors were gradually increased, such that HRT were gradually reduced and organic loading rates (OLR) were gradually increased (Table 1). The HRT in the HU reactor was set to 4.6 h, corresponding to $25\ gCOD\ L^{-1}\ d^{-1}$ OLR. Some operating problems resulted in unstable operation and variable HRT during the acclimation periods (Table 1). Therefore, the HU reactor performance was evaluated from Phase 1 of operation.

Up to Phase 1, the same feeding pump was used for the MF and SSF reactors. However, due to the lower working volume of the MF reactor along with the higher flow in the HU reactor, the OLR of the two-stage system was higher than that of the single-stage system. From Phase 2

Table 1 | Operating parameters

Systems		2-Stage system					1-Stage system		
		HU reactor		MF reactor		HU + MF reactors	SSF reactor		
Phase	Time (days)	S_0 (gCOD L^{-1})	HRT (h)	S_0 (gCOD L^{-1})	HRT (h)	OLR (gCOD L^{-1} d^{-1})	S_0 (gCOD L^{-1})	HRT (h)	OLR (gCOD L^{-1} d^{-1})
Aclim. 1	0–17	4.5	4.2	0.7	5.4	3.5	0.9	8.7	2.5
Aclim. 2	18–38	4.7	7.7	1.2	8.7	3.1	1.5	14.4	2.5
1	39–58	4.6	4.8	3.9	29.8	3.2	4.6	47.6	2.3
2	59–93	4.8	4.7	3.9	48.5	2.2	4.7	56.5	2.0
3	94–101	4.6	4.4	4.9	36.0	2.7	4.9	40.8	2.9
4	102–129	5.0	4.6	4.7	29.4	3.5	4.9	34.0	3.5
5	130–136	4.8	4.5	5.0	24.5	4.0	5.1	34.0	3.6
6	137–156	4.7	4.4	4.8	20.4	4.6	4.8	34.0	3.4
7	157–178	4.8	4.5	4.4	13.7	6.4	4.9	22.8	5.2

onward, two feeding pumps were used for the MF and SSF reactors, allowing the control of the HRT of each reactor independently. OLR was then progressively increased, by reducing the HRT in the MF and SSF reactors, according to the reactors' soluble COD removal efficiencies. In Phases 5 and 6, the HRT was reduced only in the MF reactor due to the higher efficiency of COD removal (Section 'Organic matter removal and volatile suspended solids'). Since the MF and SSF reactors reached stability during operation, HRTs were greatly reduced in Phase 7 to evaluate the impacts on their performances.

Analyses

Biogas flow rate was measured using Milligas counter gas meters (Ritter®). The H_2, CH_4 and CO_2 composition was analysed using a Shimadzu GC-2010 gas chromatograph, with the following specifications: thermal conductivity detector; argon as the carrier gas; Carboxen 1010 capillary column; initial detector and injector temperatures of 200 and 230 °C, respectively; oven temperature of 130–135 °C; flow rate of 12 mL min^{-1}; and sample volume of 300 µL.

Sucrose (glucose and fructose) and organic acids (lactic, formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric) were determined using Shimadzu System UV/DAD (210 nm) high performance liquid chromatography with Refractive Index (in series) detectors, Aminex HPX-87H column, 0.005 M H_2SO_4 solution as eluent, flow rate of 0.5 mL min^{-1}, oven temperature of 43 °C, and sample injection of 100 µL.

Total COD of the affluent, soluble COD of the effluent (filtered using 1.2 µm membrane) and volatile suspended solids (VSS) concentration in the effluent were analysed according to APHA *et al.* (2005). The pH was measured using a pH meter (Hach equipment). Total volatile acids (TVA) as well as total alkalinity, volatile acid alkalinity and bicarbonate alkalinity (BA) were measured by titration using the Kapp method (Equations (1)–(4)), as this method is considered robust and reliable (Mota *et al.* 2015).

$$TA\ (mg\ CaCO_3.L^{-1}) = \frac{Vol.H_2SO_{4\,pH\,4.3} \times N\,H_2SO_4 \times 50000}{sample\ volume} \quad (1)$$

$$TVA\ (mg\ HAc.L^{-1}) = \frac{Vol.H_2SO_{4pH\,5to4} \times N\,H_2SO_4 \times 131340}{sample\ volume} - (0.0616 \times TA) - 10.9 \quad (2)$$

$$VAA\ (mg\ CaCO_3.L^{-1}) = 0.5\ \times TVA \quad (3)$$

$$BA\ (mg\ CaCO_3.L^{-1}) = TA - VAA \quad (4)$$

DNA sequencing analyses

Sludge from the bottom and middle of the reactors was sampled at the conclusion of the operation, and genomic DNA was extracted using the Griffiths *et al.* (2000) protocol. Sequencing analyses of the 16 S rDNA gene V4-5 region were performed according to Mota *et al.* (2018), adapted from Venkiteshwaran *et al.* (2016).

RESULTS AND DISCUSSION

Hydrodynamics

Tests were performed to verify the hydrodynamic conditions in the SSF, MF and HU reactors. The reactors had not yet been inoculated, and the MF and SSF reactors were already filled with the support material. It is worth noting that the working volume used in the HRT calculations was the empty space of the reactors, excluding the support material. However, the working volume considered in the operation was the total internal volume. The reason for this difference lies in the practical concern about working volume, when the systems are compared in terms of footprint and required reactor volume; and in the difficulty of measuring the real working volume during operation. This difficulty is due to the volume occupied by the biomass. However, for hydrodynamic purposes, empty space is more appropriate for evaluating the flow regime and the existence of dead zones.

Table 2 shows the summary of hydraulic parameters. The results indicate that every reactor behaves almost as a plug-flow reactor, presenting a very low degree of mixing. The HRT curve of the HU reactor shows a clear pattern of plug flow regime. The HRT curves of the MF and SSF reactors were adjusted to the SGompertz model, using the program Origin 2017.

Acid production and stability during operation

From Phase 1, in which the operating parameters of the HU reactor were set to 4.6 h HRT and 25.6 gCOD L^{-1} d^{-1} OLR, this reactor presented great stability, and constant production of hydrogen and organic acids during operation. Although the influent pH was 6.5, the average effluent pH was 2.7. This occurred because no buffer agent was added; and, due to the constant organic acid and CO_2 production, pH values dropped below 3.0. The low pH values, along with low HRT and high OLR, completely inhibited the methanogenic microorganisms, which were not found in the HU sludge by the end of operation. The extreme acid environment did not harm H_2 production, which was stable and in a high range, corresponding to hydrogen yield of 175 ml H_2 $g^{-1}COD_{added}$. Eighty-one per cent of sucrose was removed, and, among the organic acids produced, most of the acidified sucrose was converted to acetate (54.9% molar ratio) followed by lactate (25.4% molar ratio). Minor amounts (molar ratio) of butyrate (7.7%), propionate (7.7%), valerate (2.8%) and formate (1.4%) were found. The HU reactor performance is discussed extensively in Mota *et al.* (2018), in which the HU reactor is named the UF-2 reactor.

In the MF and SSF reactors, influent and effluent pH values were kept close to neutral. Although the concentrations of the sodium bicarbonate added to the feeding of both reactors were equal, the influent pH values in the MF and SSF reactors were 6.6 and 7.8, respectively. This is due to the fact that the influent in the MF reactor contained high concentrations of organic acids. However, the differences between the influent pH values were not reflected in the effluent pH values of the MF and SSF reactors, which averaged 7.1 and 7.0, respectively. Values of pH and concentrations of BA in the effluent are shown in Figure 2.

Figure 3 shows the values of the TVA, and of each acid in the MF and SSF reactors. During acclimation period 1, the effluent of the SSF reactor showed lower concentrations of TVA and higher concentrations of BA than the effluent of the MF reactor. This was likely due to the lower OLR applied to the SSF reactor compared to the OLR applied to the two-stage system (Table 1). The increased concentration of organic matter in the feeding during acclimation period 2 caused the concentration of TVA to increase, especially in the SSF reactor, although the OLR did not change significantly. From day 25 to day 27, the MF and SSF reactors were fed only with water, sodium bicarbonate, and nutrients. This was done to avoid accumulation of TVA and, eventually, collapse of the systems.

Following acclimation, the initial organic concentration of the influent of the SSF reactor was raised to the same initial concentration of the influent of the HU reactor (approximately 4.8 gCOD L^{-1}). Also, the MF reactor started to be fed with the acidified effluent without dilution, from the HU reactor. Eventually, an increase in TVA concentrations was observed during Phase 1, despite the unchanged OLR (Table 1). As the TVA levels were above

Table 2 | Hydraulic parameters

Reactor	HRT_m[a] (h)	HRT_r[b] (h)	HRT_m/HRT_r	D/uL[c]	N[d]	R^2[e]
HU reactor	3.11	3.02	1.03	0.0032	158.0	0.99982
MF reactor	4.88	4.23	1.15	0.0188	26.6	0.99895
SSF reactor	8.08	7.46	1.08	0.0181	27.6	0.99892

[a]HRT_m: mean HRT, calculated from the division of the reactor working volume by the flow rate.
[b]HRT_r: real HRT, obtained by the hydrodynamic tests.
[c]D/uL: dimensionless dispersion number (D = longitudinal dispersion coefficient that characterizes the degree of backmixing during flow, u = fluid velocity, L = reactor length).
[d]N: number of reactors in series.
[e]Adjusted R-Square (nonlinear curve SGompertz).

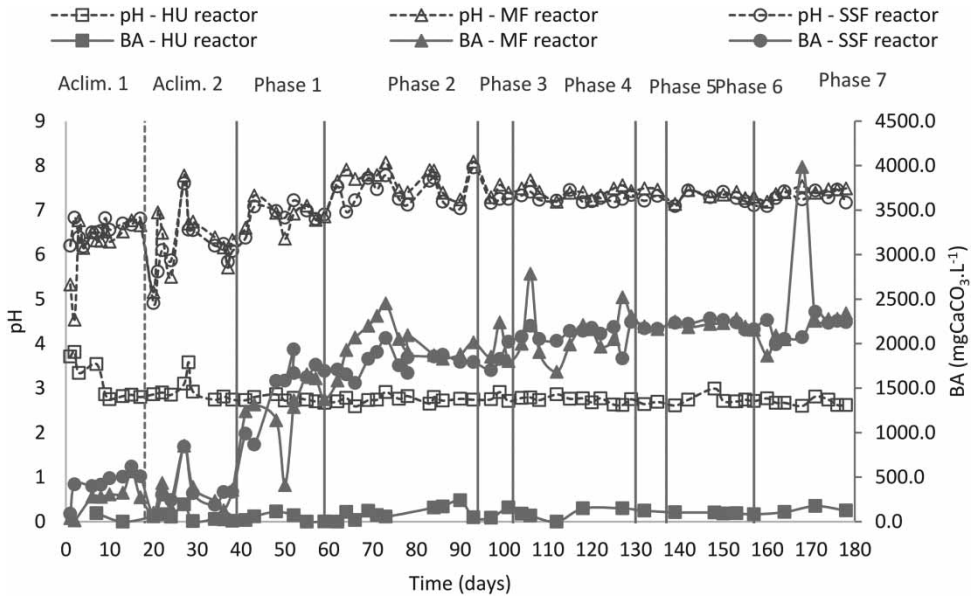

Figure 2 | pH values and bicarbonate alkalinity (BA) concentrations in the effluents of the HU, MF and SSF reactors.

1,000 mg L^{-1}, which is the inhibitory threshold for the methanogenic archaea (Foresti 2002), the OLR was reduced in Phase 2 by increasing the HRT of the MF and SSF reactors. This procedure effectively reduced the TVA concentrations. During this stage, in which the flow rates were adjusted in order to keep the HRT equal between the two- and single-stage systems (Table 1), the MF reactor started to show superior performance. This can be observed from its lower TVA concentrations.

The increased OLR in Phases 3 and 4 did not impact the performance of the reactors. This suggests that the high TVA concentrations observed previously may have resulted from the shock loading, and from kinetic limitations due to the slow growth of methanogenic archaea. The TVA concentrations usually remained lower than 500 mg HAc L^{-1}. The non-correlation between the sum of the acid species and the TVA from Phase 3 to Phase 7 (Figure 3(b)), probably is due to the inaccuracy of the titration method when the organic acid concentrations are low in relation to the bicarbonate concentrations (Mota *et al.* 2015). BA was above 1,700 mg $CaCO_3$ L^{-1} and the IA:PA ratio was below 0.5 in the effluent from both reactors, from Phase 3 onward, indicating good stability of the systems. Since the TVA and COD levels were lower in the effluent of the MF reactor during Phases 2, 3 and 4, a higher OLR was applied in the two-stage system, compared to the single-stage system, in Phases 5, 6 and 7 (Table 1). In Phase 7, the OLR in the MF and SSF reactors was increased by approximately 50%, in relation to the previous phase. The two-stage system (HU + MF) and the MF reactor operated at 6.4 and 7.7 gCOD L^{-1} d^{-1} OLR, respectively. The SSF reactor operated at 5.2 gCOD L^{-1} d^{-1} OLR. However, the impacts on performance were mild, suggesting that the methanogenic community was established in the reactors.

Regarding the acid species, the increased influent concentration in Phase 1 resulted in an accumulation of acetate, propionate, butyrate and valerate. The OLR reduction in Phase 2 resulted in the reduced acid concentrations. However, it was in Phase 3 that the concentrations dropped substantially, to levels below 180 mg L^{-1}. Lactate, formate, and (iso)valerate were no longer detected. Very low concentrations (<11 mg L^{-1}) of butyrate were detected only in the SSF reactor. Higher levels of acetate and propionate were detected, suggesting kinetic limitations of acetoclastic methanogenesis and disturbances in the systems. As pointed out by Kim *et al.* (2002), TVA degradation, especially propionate, can be hampered by relatively low concentrations of dissolved hydrogen and acetate, also by hydrogen interspecies transfer limitations. It was demonstrated that, during the stable periods (Phases 3 to 7), acid concentrations in the effluent of the MF reactor were below those found in the effluent the SSF reactor. This was true even in the periods when the OLR applied in the two-stage system was the highest. These results suggest that the separation of anaerobic digestion phases reduced the problems related to kinetic, thermodynamic and mass transfer limitations, under these conditions.

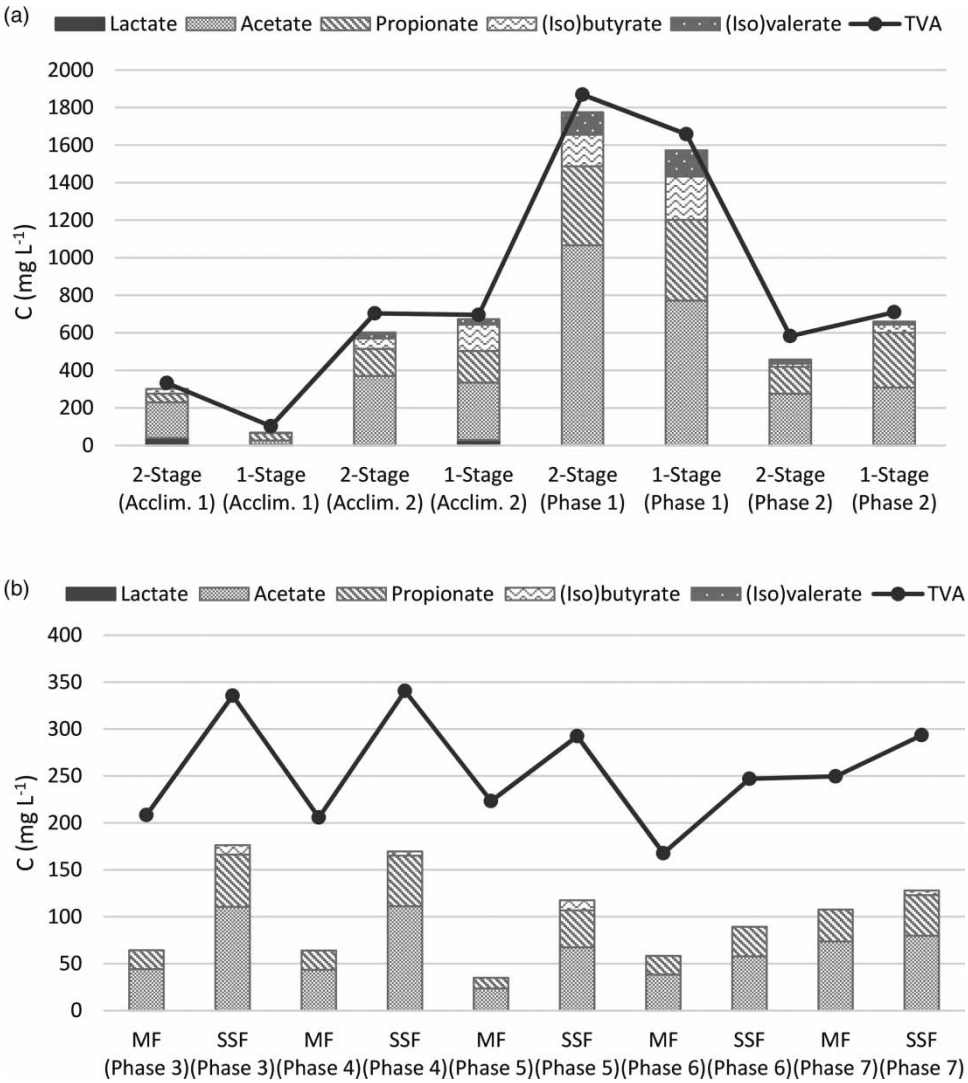

Figure 3 | Organic acid concentrations in the final effluent of the two-stage (MF reactor) and single-stage (SSF reactor) systems during start-up (a) and stable reactors performances (b).

Organic matter removal and volatile suspended solids

Soluble effluent COD levels showed the same trends observed for TVA levels. During start-up, influent dilution was not sufficient to achieve satisfactory performance and effluent COD levels were quite high. Means of 484 mg L^{-1} were found in the MF reactor, and of 184 mg L^{-1} in the SSF reactor. The 5- to 3.3-fold reduction, during acclimation period 2, resulted in even higher effluent COD levels, which increased two-fold in the MF reactor (mean of 1,028 mg L^{-1}) and nine-fold in the SSF reactor (mean of 1,200 mg L^{-1}) (excluding days 25–27, when the feed was only water and nutrients).

Feeding the reactors with undiluted influent during Phase 1 caused an increase in effluent COD (Figure 4). Acidification of the MF and SSF reactors was probably a key factor in their poor performance. Mean COD removal efficiencies in the two-stage system (HU + MF) and in the SSF reactor were only 43.3% and 51.8%, respectively. During Phase 2, when the HRTs in the MF and SSF reactors were increased, better COD removal efficiencies were achieved, corresponding to 85.8% and 79.8% in the two- and single-stage systems, respectively. From Phase 3 onward, reactors reached stability and the total effluent COD was also monitored. From Phase 2 to Phase 4, the two- and single-stage systems operated under equivalent OLR (Table 1). Under these conditions, the two-stage system achieved a higher COD removal efficiency than the SSF reactor. Therefore, in Phases 5 and 6, the OLR was increased (by reducing the HRT) only in the MF reactor.

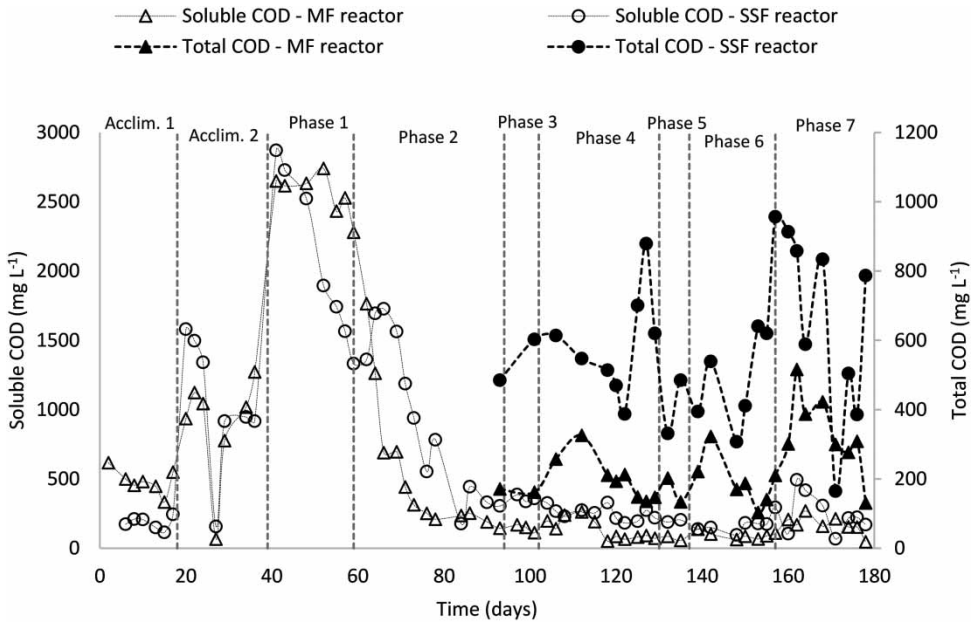

Figure 4 | COD levels in the final effluent of the two-stage (MF reactor) and single-stage (SSF reactor) systems.

This was done to evaluate if the OLR could be increased in the two-stage system, while maintaining the same or higher COD removal efficiency as that of the SSF reactor.

The progressive increase of OLR, by approximately 20%, in the MF reactor at each operational phase until Phase 6 did not affect its performance (Figure 4). The removal of soluble and total COD in the two-stage system increased from 85.8% and 76.1% during Phase 2, to 98.1% and 96.0% during Phase 6, respectively. During these periods, soluble and total COD removal in the SSF reactor were 79.8% and 77.8% (Phase 2), and 96.8% and 89.9% (Phase 6), respectively. Since the MF and SSF reactors achieved stability, as indicated by the effluent COD levels from Phase 3 to Phase 6, the OLR was increased by 50% in Phase 7. The greater increase of OLR during Phase 7 caused a slight reduction in COD removal. Total COD removal in the two-stage system decreased from 96.0% to 93.4%, and in the SSF reactor it decreased from 89.9% to 86.6%.

From Phase 3 to 7, the non-acidified soluble organic matter, i.e. total soluble COD minus the COD from the organic acids (detected by chromatography), was approximately 40 mg L^{-1} in the MF reactor and 70 mg L^{-1} in the SSF reactor. Higher levels of non-acidified soluble COD in the SSF reactor, in relation to the MF reactor, indicate higher concentration of soluble microbial products (SMP) and extracellular polymeric substances (EPS). Studies indicate that the microorganisms involved in the acidogenic stage release more SMP into the medium than those involved in the methanogenic stage (Jeison 2007; Wu & Zhou 2010; Mota *et al.* 2013). Thus, it is believed that the higher COD levels, related to SMP and/or EPS, were due to higher acidogenic bacteria growth in the SSF reactor.

Increased total COD levels in the effluent of the SSF reactor resulted not only from soluble COD, but especially from particulate COD, i.e. total COD minus soluble COD. This is corroborated by its highest VSS concentrations (Figure 5). The particulate COD/VSS ratio was 1.30 in the MF reactor and 1.46 in the SSF reactor, which is close to the theoretical value of 1.42 gO_2 per g of biodegradable VSS (von Sperling & Chernicharo 2005). The lower COD/VSS ratio in the MF reactor could be due to a higher degree of stabilization of the biomass. Parker *et al.* (2008) observed a reduction of the sludge COD/VSS ratio, from 1.45 to 1.2, after 33 days of anaerobic digestion.

The increased VSS concentrations in the effluent of the SSF reactor (223 ± 119 mg L^{-1}) were partially attributed to the fast growth rates of acidogenic bacteria, and increased EPS adhering to the microbial flocs. On the other hand, VSS concentrations in the effluent of the MF reactor were much lower (80 ± 36 mg L^{-1}), despite the high concentrations in the feeding, that is, the effluent of the HU reactor (257 ± 167 mg L^{-1}). It is believed that, as a result of the low availability of non-acidified substrate, acidogenic bacteria from the HU reactor underwent decay when they entered the MF reactor, culminating in the stabilization of those bacteria as well as of the EPS and SMP released by them. In contrast, since there was a constant inflow of non-acidified substrate in the SSF reactor, the acidogenic

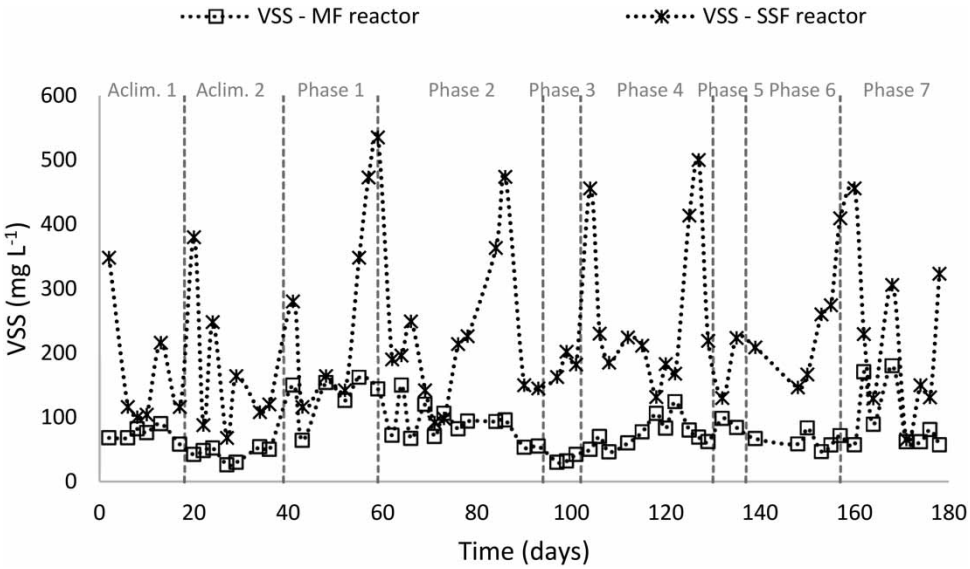

Figure 5 | VSS concentrations in the final effluent of the two-stage system (MF reactor) and of the single-stage system (SSF reactor).

bacteria were continuously growing and releasing SMP and EPS. This resulted in higher concentrations of COD and VSS in the final effluent.

Biogas composition and production

From Phase 1 onwards, biogas from the HU reactor presented H_2 content of $59.8 \pm 5.9\%$ and CO_2 content of $40.5 \pm 5.9\%$. CH_4 was not detected, which led to the assumption that the environmental conditions established by the pH self-adjustment and low HRT were sufficient to completely inhibit methanogenesis (Mota *et al.* 2018).

Biogas from the MF and SSF reactors was composed of CH_4, CO_2, and, during the instability periods, traces of H_2 (Figure 6). During acclimation 1, the biogas from the SSF reactor presented higher CH_4 content (71.6%) and lower H_2 content (0.3%) than the biogas from the MF reactor (62.2% CH_4 and 3.3% H_2). This was probably due to the lower OLR applied in the SSF reactor. During acclimation 2, the increase in the COD concentration in the influent

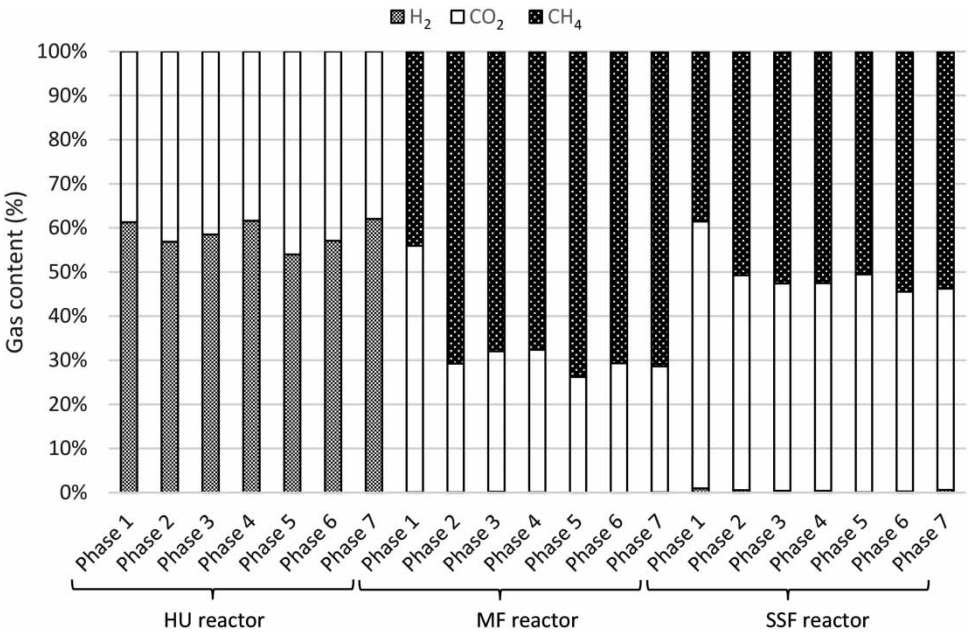

Figure 6 | Biogas composition.

had a strong impact on the composition of the biogas from the SSF reactor, which initially presented 58.5% CO_2, 31.7% H_2, and only 9.8% CH_4. However, the SSF reactor recovered methanogenic activity during the course of operation and, at the end of acclimation 2, H_2 content had decreased to 0.3% and CH_4 content had increased to 41.1%. The composition of the biogas from the MF reactor did not suffer major disturbances because of increased concentration in the influent. CH_4 content was approximately 56.4% and H_2 concentration dropped to null values, by the end of the acclimation.

The increase in the organic concentration of the influent in Phase 1 led to a reduction in CH_4 content in the biogas from the MF reactor, averaging 44.0%. The H_2 content remained negligible (max. 0.2%), indicating that methanogenic microorganisms had been established. The CH_4 content initially decreased to 32.6% in the SSF reactor. However, it increased throughout the operation, and presented an average of 38.5% in Phase 1. It is notable that, in Phase 2, the CH_4 content of the biogas from the MF reactor remained much higher than the biogas from the SSF reactor (Figure 6). This suggests a higher calorific potential of the biogas from the MF reactor. These results are consistent with results from other studies that also compared two- to single-stage anaerobic digestion, in which increased CH_4 content was found in the methanogenic reactor fed with acidified wastewater (Ghosh *et al.* 1985; Yeoh 1997). It is assumed that the occurrence of acidogenesis in a previous stage resulted in lower CO_2 production in the MF reactor, resulting in higher CH_4 content in the biogas.

Figure 7 shows the volumetric methane production rates (VMPH) when the MF and SSF reactors had reached stability. Results were gathered according to the operating conditions: Phases 3 and 4, in which the systems were under the same OLR; Phases 5 and 6, in which the two-stage system was under higher OLR than the single-stage system; and Phase 7, in which OLR was greatly increased in both systems. Since Phases 3 and 5 lasted only a few days, and the OLR increases were mild from Phase 3 to Phase 4 and from Phase 4 to Phase 5, minor changes in the VMPH occurred between these phases. To calculate the VMPR in the two-stage system, two scenarios were considered: one refers to the VMPR in the MF reactor specifically; the other refers to the VMPR in the two-stage system, taking the volume of the HU reactor into account. In the latter case, the HU volume considered was proportional to the flow into the MF reactor.

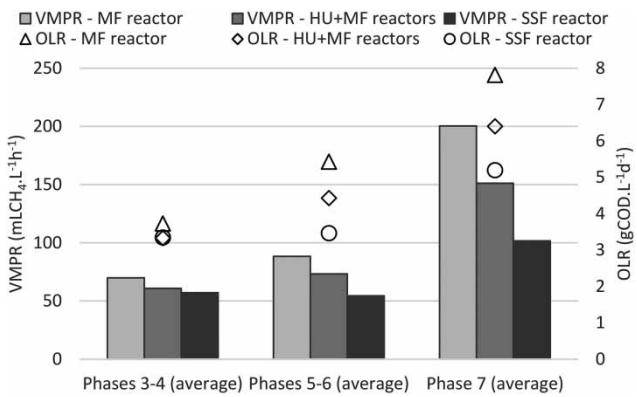

Figure 7 | Volumetric methane production rate in the single- and two-stage systems.

In Phases 3 and 4, no differences in the VMPR were observed. The VMPR corresponded to a mean of 61 and 57 $mLCH_4 \cdot L^{-1} h^{-1}$ in the two- and single-stage systems, respectively. Therefore, under these conditions, the two-stage system offered no advantage over the single-stage system, regarding the VMPR parameter. In fact, the methane yield was also very similar in both systems during Phase 4, as discussed in Section 'Treatment efficiency and energy yield in two- and single stage systems' (Table 4). Naturally, the increase in OLR resulted in an increase in VMPR. In Phase 7, the highest VMPRs were achieved in both systems, 151 and 102 $mLCH_4 \cdot L^{-1} h^{-1}$ in the two- and single-stage systems, respectively. It is noteworthy that the higher VMPR in the two-stage system in Phase 7 was not only due to the higher OLR, but also to the higher methane yield during this period (Table 4).

Treatment efficiency and energy yield in two- and single-stage systems

Data from the two- and single-stage systems were analysed, for the purpose of performance comparison. Data from Phase 4, in which the same OLR of 3.5 $gCOD\ L^{-1} d^{-1}$ was applied in both systems, and data from Phase 7, in which the systems operated at their respective maximum OLRs, corresponded to 6.4 in the two-stage system and 5.2 $gCOD\ L^{-1} d^{-1}$ in the single-stage system.

Treatment performance data are shown in Table 3. These data show that superior performance was achieved in the two-stage system for all parameters analysed, especially total COD and VSS. These results reinforce the possibility of achieving higher COD removal efficiencies and/or higher OLR in two-stage systems, as reported in previous studies (Cho 1983; Azbar & Speece 2001; Nasr *et al.* 2012; Ferraz Júnior *et al.* 2016).

Suitable environmental conditions for methanogens, such as neutral pH, high BA (>2,000 $mg\ L^{-1}$), low

Table 3 | Treatment efficiency in Phases 4 and 7

	Phase 4[a]		Phase 7[b]	
Effluent quality	**2-stage**	**1-stage**	**2-stage**	**1-stage**
pH	7.4	7.3	7.4	7.3
TVA (mg HAc L^{-1})	205.7	340.7	249.6	293.5
Total COD (mg L^{-1})	203.9	591.5	316.9	665.8
Soluble COD (mg L^{-1})	134.9	252.1	164.7	255.6
VSS (mg L^{-1})	75.2	265.5	92.1	244.4
Total COD removal (%)	95.9	87.7	93.4	86.6
Soluble COD removal (%)	97.3	94.9	96.6	94.8

[a]OLR (gCOD L^{-1} d^{-1}): HU reactor = 25.8, MF reactor = 3.9, HU + MF reactor = 3.5, SSF reactor = 3.5.
[b]OLR (gCOD L^{-1} d^{-1}): HU reactor = 26.0, MF reactor = 7.8, HU + MF reactor = 6.4, SSF reactor = 5.2.

concentrations of TVA (<500 mg L^{-1}), temperature of 30 °C, and no affluence of toxic substances, were maintained in both the MF and SSF reactors. Therefore, it is believed that the main factors contributing to the better performance of the two-stage system were: (i) lower growth of acidogenic bacteria in the MF reactor, resulting in lower COD and VSS concentrations in the final effluent; (ii) withdrawal of H_2 produced in the acidogenic step, contributing to overcoming the thermodynamic limitations on the acetogenic reactions; and (iii) high production of acetic acid in the HU reactor, reducing the dependence on syntrophic bacterial activity for the conversion of organic matter into biogas in the MF reactor.

Finally, the energy yields obtained during the treatment were estimated (Table 4). In an attempt to normalize the input parameters, the COD added at the entrance of the systems, corresponding to the initial concentration of the raw wastewater, was considered. In order to display the results in energy units, a calorific potential of 142 kJ per g of H_2 and 50 kJ per g of CH_4 (Nasr *et al.* 2012) were considered. In the single-stage system, the total energy yield was estimated in the range from 12.78 kJ/$gCOD_{added}$ to 15.49 kJ/$gCOD_{added}$, with the yield from H_2 production being negligible. In the two-stage system, the total energy yield was estimated in the range from 16.02 to 20.69 kJ/$gCOD_{added}$, with approximately 2.2 kJ/$gCOD_{added}$ coming from the H_2 produced in the acidogenic reactor. Similar results were obtained by Luo *et al.* (2011). They found the yield in the two-stage system was 13.1 kJ per g of VS added (12.4 kJ from CH_4 and 0.7 kJ from H_2); and the yield in the single-stage system was 11.8 kJ per g of VS added.

Under the conditions evaluated, the replacement of a single-stage system by a two-stage system can result in energy yield increases of 25.3% (Phase 4) and 33.6% (Phase 7). Even excluding H_2 recovery, the estimated energy yields from the two-stage system were 7.7% and 20.7% higher in Phases 4 and 7, respectively, in relation to the single-stage system. Mamimin *et al.* (2015) reported 34% higher methane yield in a UASB fed with acidified palm oil mil effluent compared to a UASB fed with raw effluent.

Due to the relatively low energy yield from H_2 production, from 10.3 to 14.3% of the total energy, a possible use of the H_2 is as a combustion catalyst rather than as an energy carrier. For this purpose, the biogas from the acidogenic reactor can be mixed with the biogas from the methanogenic reactor, producing a mixture of CH_4, CO_2 and H_2. This mixture, sometimes called bio-hythane, may offer advantages, such as improved combustion properties and reduced CO_2 and NO_X emissions, over conventional biogas consisting of only CH_4 and CO_2 (Ortenzi *et al.* 2008; Ghoniem 2011; Cavinato *et al.* 2012).

Table 4 | Energy yields in Phases 4 and 7

	Phase 4[a]		Phase 7[b]	
Energy yield	**2-stage**	**1-stage**	**2-stage**	**1-stage**
HY (kJ H_2/$gCOD_{added}$)	2.29	0.03	2.14	0.12
MY (kJ CH_4/$gCOD_{added}$)	13.73	12.75	18.55	15.36
EY (kJ H_2 + kJ CH_4/$gCOD_{added}$)	16.02	12.78	20.69	15.49
HY/EY	14.3%	0.3%	10.3%	0.8%
MY/EY	85.7%	99.7%	89.7%	99.2%

HY, hydrogen energy yield; MY, methane energy yield; EY, total energy yield.
[a]OLR (gCOD L^{-1} d^{-1}): HU reactor = 25.8, MF reactor = 3.9, HU + MF reactor = 3.5, SSF reactor = 3.5.
[b]OLR (gCOD L^{-1} d^{-1}): HU reactor = 26.0, MF reactor = 7.8, HU + MF reactor = 6.4, SSF reactor = 5.2.

Microbial identification

A total of 35,665, 31,617 and 32,400 sequences were obtained from the inoculum, from the MF and SSF reactors, respectively. Archaea dropped from 9.7% in the inoculum to 2.3% and 1.2% in the MF and SSF reactors, respectively. Overloading of the systems during start-up probably affected archaea survival, as indicated by the high organic acid concentrations in the effluent. Archaea were less than 0.1% in the HU reactor, and two bacterial sequences affiliated with *Ethanoligenens* and *Clostridium* accounted for 96% of the microbiota (Mota *et al.* 2018).

Microbial diversity was higher in the MF and SSF reactors. Microbial composition of the main sequences is shown in Table 5. Regarding archaea composition, it is noteworthy that the hydrogenotrophic methanogens belonging to the

Table 5 | Comparative study of 16 S rDNA sequencing (V4-5 region) using SINA (v1.2.11)

Domain	Otu	Phylum	Class	Order	Family	Genus	Inoculum	MF	SSF
Archaea	Otu002	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosaetaceae	*Methanosaeta*	49%	49%	19%
	Otu004	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanobacterium*	13%	14%	10%
	Otu001	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanobacterium*	7%	12%	13%
	Otu003	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanobacterium*	0%	6%	11%
	Otu010	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	*Methanosarcina*	0%	5%	4%
	Otu007	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanospirillaceae	*Methanospirillum*	0%	3%	13%
	Otu019	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanospirillaceae	*Methanospirillum*	0%	0%	22%
Bacteria	Otu0021	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	*Veillonella*	0%	14%	1%
	Otu0012	unclassified	unclassified	unclassified	unclassified	unclassified	0%	7%	17%
	Otu0042	Chlorobi	Ignavibacteria	Ignavibacteriales	unclassified	unclassified	0%	7%	0%
	Otu0028	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Lactococcus*	0%	6%	2%
	Otu0020	Firmicutes	Clostridia	Clostridiales	Clostridiaceae_1	*Clostridium_sensu_stricto_1*	1%	6%	3%
	Otu0018	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	unclassified	0%	1%	17%
	Otu0033	unclassified	unclassified	unclassified	unclassified	unclassified	0%	0%	8%
	Otu0045	SHA – 109	unclassified	unclassified	unclassified	unclassified	0%	0%	6%

Relative abundance >5% for each domain in the MF and SSF reactors.

genus *Methanospirillum* were abundant only in the SSF reactor. This is an indication of hydrogen availability, from fermentative activity. Since sucrose is a very easily biodegradable substrate, it is likely that hydrogen accumulated on the bottom of the SSF reactor, favouring the growth of hydrogenotrophic methanogens. However, the relative abundance of *Methanobacterium*, also a genus of hydrogenotrophic methanogens (Chernicharo 2007), was very similar between the reactors. On the other hand, the genus *Methanosaeta*, that produces methane exclusively from acetate (Chernicharo 2007), presented a higher relative abundance in the MF reactor. This suggests that the feeding with the acetate-rich substrate favoured their maintenance.

No marked differences were observed regarding bacterial composition. In both reactors, bacteria from the Veillonellaceae family constituted the group found most abundantly. The ability of the genus *Veillonella* to ferment lactate has been reported (Madigan *et al.* 2015). Hence, the presence of these bacteria may have been due to the high concentrations of lactate, which is a common product of sucrose fermentation.

CONCLUSIONS

Following start-up, and having achieved stable performance, the quality of the final effluent from the two-stage system was superior to that from single-stage system. At the end of operation, total COD removal was 93% and 87%, and effluent VSS concentration was 92 and 244 mg L^{-1}, in the two- and single-stage systems, respectively. The two-stage system also demonstrated higher potential for bioenergy production. Biogas from the two-stage methanogenic reactor presented 70% methane, in contrast to biogas from the single-stage system that presented 52% methane. In addition, the two-stage system provided increased energy yields up to 34% through methane and hydrogen production. Hydrogenotrophic methanogens belonging to the genus *Methanospirillum* were the most found in the single-stage reactor. Acetoclastic methanogens belonging to the genus *Methanosaeta* were the most found in the methanogenic reactor of the two-stage system. An outcome of the positive results from this and comparative research is that two-stage anaerobic reactors should be considered as an alternative for wastewater treatment systems.

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REFERENCES

APHA, AWWA, WEF 2005 *Standards Methods for the Examination of Water and Wastewater*, 21st edn. American Public Health Association/ American Water Works Association/Water Environmental Federation, Washington, DC, USA.

Azbar, N. & Speece, R. E. 2001 Two-phase, two-stage, and single-stage anaerobic process comparison. *Journal of Environmental Engineering* **127**, 240–248.

Cavinato, C., Giuliano, A., Bolzonella, D., Pavan, P. & Cecchi, F. 2012 Bio-hythane production from food waste by dark fermentation coupled with anaerobic digestion process: a long-term pilot scale experience. *International Journal of Hydrogen Energy* **37** (15), 11549–11555.

Chernicharo, C. A. L. 2007 *Anaerobic Reactors: Biological Wastewater Treatment Series*, Vol. 4, 1st edn. IWA Publishing, London, UK.

Cho, Y. K. 1983 Performance of a two-stage methane digester for alcohol stillage derived from sugarcane molasses. *Biotechnology Letters* **5** (8), 555–560.

Cohen, A., Breure, A. M., Van Andel, J. G. & Van Deursen, A. 1982 Influence of phase separation on the anaerobic digestion of glucose, 2. Stability and kinetic responses to shock loadings. *Water Research* **16**, 449–455.

Ferraz Júnior, A. D. N., Koyama, M. H., de Araújo Júnior, M. M. & Zaiat, M. 2016 Thermophilic anaerobic digestion of raw sugarcane vinasse. *Renewable Energy* **89**, 245–252.

Foresti, E. 2002 Anaerobic treatment of domestic sewage: established technologies and perspectives. *Water Science and Technology* **45** (10), 181–186.

Fuess, L. T., Kiyuna, L. S. M., Ferraz Júnior, A. D. N., Persinoti, G. F., Squina, F. M., Garcia, M. L. & Zaiat, M. 2017 Thermophilic two-phase anaerobic digestion using an innovative fixed-bed reactor for enhanced organic matter removal and bioenergy recovery from sugarcane vinasse. *Applied Energy* **189**, 480–491.

Ghoniem, A. F. 2011 Needs, resources and climate change: clean and efficient conversion technologies. *Progress in Energy and Combustion Science* **37** (1), 15–51.

Ghosh, S., Ombregt, J. P. & Pipyn, P. 1985 Methane production from industrial wastes by two-phase anaerobic digestion. *Water Research* **19** (9), 1083–1088.

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G. & Bailey, M. J. 2000 Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environmental Microbiology* **66** (12), 5488–5491.

Jeison, D. 2007 *Anaerobic Membrane Bioreactors for Wastewater Treatment: Feasibility and Potential Applications*. PhD Thesis (Educational Program of SENSE) – Netherlands, Research School for the Socio-Economic and Natural Sciences of the Environment, Wageningen University, the Netherlands.

Ke, S., Shi, Z. & Fang, H. H. P. 2005 Applications of two-phase anaerobic degradation in industrial wastewater treatment. *International Journal of Environment and Pollution* **23**, 65–80.

Kim, M., Ahn, Y. H. & Speece, R. E. 2002 Comparative process stability and efficiency of anaerobic digestion; mesophilic vs thermophilic. *Water Research* **36**, 4369–4385.

Kim, S. H., Han, S. K. & Shin, H. S. 2004 Two-phase anaerobic treatment system for fat-containing wastewater. *Journal of Chemical Technology and Biotechnology* **79** (1), 63–71.

Levenspiel, O. 1999 *Chemical Reaction Engineering*, 3rd edn. John Wiley & Sons Inc., New York, NY, USA.

Lullio, T. G., Souza, L. P., Ratusznei, S. M., Rodrigues, J. A. D. & Zaiat, M. 2014 Biomethane production in an AnSBBR treating wastewater from biohydrogen process. *Applied Biochemistry and Biotechnology* **174**, 1873–1896.

Luo, G., Xie, L., Zhou, Q. & Angelidaki, I. 2011 Enhancement of bioenergy production from organic wastes by two-stage anaerobic hydrogen and methane production process. *Bioresource Technology* **102**, 8700–8706.

Ma, H., Ye, L., Hu, H., Zhang, L., Ding, L. & Ren, H. 2017 Determination and variation of core bacterial community in a two-stage full-scale anaerobic reactor treating high-strength pharmaceutical wastewater. *Journal of Microbiology and Biotechnology* **27** (10), 1808–1819.

Madigan, M. T., Martinko, J. M., Bender, K. S., Buckley, D. H. & Stahl, D. A. 2015 *Brock Biology of Microorganisms*, 14th edn. Pearson, Boston, MA, USA.

Mamimin, C., Singkhala, A., Kongjan, P., Suraraksa, B., Prasertsan, P., Imai, T. & O-Thong, S. 2015 Two-stage thermophilic fermentation and mesophilic methanogen process for biohythane production from palm oil mill effluent. *International Journal of Hydrogen Energy* **40** (19), 6319–6328.

Mockaitis, G., Pantoja, J. L., Rodrigues, J. A., Foresti, E. & Zaiat, M. 2014 Continuous anaerobic bioreactor with a fixed-structure bed (ABFSB) for wastewater treatment with low solids and low applied organic loading content. *Bioprocess and Biosystems Engineering* **37** (7), 1361–1368.

Mota, V. T., Santos, F. S. & Amaral, M. C. S. 2013 Two-stage anaerobic membrane bioreactor for the treatment of sugarcane vinasse: assessment on biological activity and filtration performance. *Bioresource Technology* **146**, 494–503.

Mota, V. T., Santos, F. S., Araújo, T. A. & Amaral, M. C. S. 2015 Evaluation of titration methods for volatile fatty acids measurement: effect of the bicarbonate interference and feasibility for the monitoring of anaerobic reactors. *Water Practice and Technology* **10** (3), 486–495.

Mota, V. T., Ferraz Júnior, A. D. N., Trably, E. & Zaiat, M. 2018 Biohydrogen production at pH below 3.0: is it possible? *Water Research* **128**, 350–361.

Nasr, N., Elbeshbishy, E., Hafez, H., Nakhla, G. & El Naggar, M. H. 2012 Comparative assessment of single-stage and two-stage anaerobic digestion for the treatment of thin stillage. *Bioresource Technology* **111**, 122–126.

Ortenzi, F., Chiesa, M., Scarcelli, R. & Pede, G. 2008 Experimental tests of blends of hydrogen and natural gas in light-duty vehicles. *International Journal of Hydrogen Energy* **33** (12), 3225–3229.

Parker, W., Jones, R. & Murthy, S. 2008 Proceedings of the Water Environment Federation, WEFTEC 2008: Session 1 through Session 10, (10), 524–533.

Perendeci, N. A., Tanyolaç, A. & Çelebi, S. S. 2012 A simplified kinetic model for a full scale anaerobic wastewater treatment

plant of a sugar factory under unsteady conditions. *Desalination and Water Treatment* **40** (1–3), 118–128.

Picanço, A. P., Vallero, M. V. G., Gianotti, E. P., Zaiat, M. & Blundi, C. E. 2001 Influence of porosity and composition of supports on the methanogenic biofilm characteristics developed in a fixed bed anaerobic reactor. *Water Science and Technology* **44** (4), 197–204.

Rapport, J., Zhang, R., Jenkins, B. M. & Williams, R. B. 2008 *Current Anaerobic Digestion Technologies Used for Treatment of Municipal Organic Solid Waste*. Report California Environmental Protection Agency, Sacramento, CA, USA.

van Groenestijn, J. W., Hazewinkel, J. H. O. & Bakker, R. R. 2006 Pre-treatment of ligno-cellulose with biological acid recycling (the biosulfurol process). *Zuckerindustrie* **131**, 639–641.

Venkiteshwaran, K., Milferstedt, K., Hamelin, J. & Zitomer, D. H. 2016 Anaerobic digester bioaugmentation influences quasi steady state performance and microbial community. *Water Research* **104**, 128–136.

Von Sperling, M. & Chernicharo, C. A. L. 2005 *Biological Wastewater Treatment in Warm Climate Regions*. IWA Publishing, London, UK.

Wu, B. & Zhou, W. 2010 Investigation of soluble microbial products in anaerobic wastewater treatment effluents. *Journal of Chemical Technology and Biotechnology* **85**, 1597–1603.

Yeoh, B. G. 1997 Two-phase anaerobic treatment of cane-molasses alcohol stillage. Anaerobic digestion VIII. *Water Science and Technology* **36** (6–7), 441–448.

Young, J. C., Kim, I. S., Page, I. C., Wilson, D. R., Brown, G. J. & Cocci, A. A. 2000 Two-stage anaerobic treatment of purified terephthalic acid production wastewaters. *Water Science and Technology* **42** (5–6), 277–282.

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